



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/52, 9/00, 15/81, 15/11, 1/19 // (C12N 1/19, C12R 1:865), C12N 15:69	A1	(11) International Publication Number: WO 99/00504 (43) International Publication Date: 7 January 1999 (07.01.99)
(21) International Application Number: PCT/GB98/01885 (22) International Filing Date: 26 June 1998 (26.06.98) (30) Priority Data: 9713412.6 26 June 1997 (26.06.97) GB (71) Applicant (for all designated States except US): DELTA BIOTECHNOLOGY LIMITED [GB/GB]; Castle Court, Castle Boulevard, Nottingham NG7 1FD (GB). (72) Inventor; and (75) Inventor/Applicant (for US only): SLEEP, Darrell [GB/GB]; Delta Biotechnology Limited, Castle Court, Castle Boulevard, Nottingham NG7 1FD (GB). (74) Agent: BASSETT, Richard; Eric Potter Clarkson, Park View House, 58 The Ropewalk, Nottingham NG1 5DD (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: IMPROVED PROTEIN EXPRESSION STRAINS (57) Abstract The use of a means to vary Ubc4p or Ubc5p activity in a fungal cell to control the copy number of a plasmid in the cell. The level of Ubc4p or Ubc5p activity may be reduced/abolished (for example by gene deletion, mutagenesis to provide a less active protein, production of antisense mRNA or production of competitive peptides) to raise the copy number and increase yield of a protein encoded by the plasmid.		

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IMPROVED PROTEIN EXPRESSION STRAINS

Field of the invention

- 5 This invention relates primarily to the development of fungal strains which express proteins at levels substantially higher than the parental strains.

Background and prior art

- 10 For some 20 years, desired foreign proteins have been produced in microorganisms. However, having introduced the necessary coding sequence and obtained expression, much still remains to be done in order to optimise the process for commercial production. One area of interest concerns strain improvement, that is to say finding or making strains of
15 the host microorganism which enable the protein to be made in higher yields or better purity, for example.

To increase the yield, once a good expression system (eg transcription promoter) has been devised, one might envisage trying to increase the
20 copy number of the coding sequence (although this will have the desired effect only if DNA transcription was the limiting factor), or to increase the stability of the mRNA or to decrease the degradation of the protein. Thus, as an example of the latter approach, yeast strains (eg *pep4-3*) which are deficient in certain proteases have been used for producing
25 desired foreign proteins. In another approach, the number of 2 μ m-based plasmids in the yeast *Saccharomyces cerevisiae* has been increased by introducing a *FLP* gene into the genome under the control of a regulated promoter, eg *GAL*. Upon switching to a growth medium containing

galactose as the sole carbon source, plasmid copy number rises (11), but the plasmid copy number increase is uncontrolled since the *GAL* promoter is not repressed by *REP1/REP2*. This leads to reduced growth rate and thence clonal selection of *cir*^o derivatives of the original *cir*⁺ strain
5 (11,20).

We have mutated yeast strains by application of mutagens in order to generate mutants randomly and thereby hopefully find mutant strains which produce heterologous proteins in better yield (16,21). We have
10 now characterised such a randomly-produced mutant which maintained a higher number of copies of the plasmid expressing the desired protein and have found that the mutation occurred in one of the genes encoding ubiquitin-conjugating enzymes, namely *UBC4*. The *UBC4*-encoded enzyme (and the closely related *UBC5*-encoded) enzyme are involved in
15 degrading aberrant and short lived proteins and there was no reason to have supposed that the deletion of either of them would have enabled an increased yield of a normal, desired, protein to have been obtained.

Several genes encoding ubiquitin conjugating enzymes (*UBC*) have been
20 implicated in the bulk protein degradation and in the stress response of yeast. *UBC1*, *UBC4* and *UBC5* act together to mediate important functions for cell growth and cell viability (2,3). Yeast strains with a mutation in a single gene are viable and have similar growth rates to the parental strains, but *ubc4/ubc5* double mutants have reduced growth rates
25 and are sensitive to amino acid analogues, while a triple mutant is inviable, indicating that their activities overlap. The *UBC4* and *UBC5* genes are closely related and the two coding DNA sequences share 77% identical residues, while the predicted amino acid sequences of the two

proteins show 92% identical residues (3). Because of the near identity of the Ubc4 and Ubc5 proteins (hereafter abbreviated to Ubc4p and Ubc5p) it has been suggested that *UBC4* could complement for the loss of function of the *ubc5* mutant and *vice versa* (3). This would explain why the dramatic reduction in growth rate was only observed in *ubc4/ubc5* double mutants. Pulse chase experiments have indicated that Ubc4p and Ubc5p are responsible for the degradation of short-lived and abnormal proteins, but that the turnover of these proteins was only reduced in strains with the *ubc4/ubc5* double mutation. It was not reduced in strains with single *ubc4* or *ubc5* mutations (3). This reference, therefore, suggested that the use of single *ubc4* and *ubc5* mutant fungal strains would not be beneficial.

Structurally, all known UBC genes encode a conserved domain (known as the UBC domain) of approximately 16kDa containing the conserved conjugating cysteine (1,22). Transfer of activated ubiquitin results in the covalent attachment of the C-terminus of ubiquitin via a thioester bond to the cysteine residue. *UBC* genes have been divided into different classes (reviewed in 22). Class I *UBC* genes are composed almost exclusively of the conserved UBC domain, class II and class III *UBC* genes have C-terminal or N-terminal extension, respectively, while class IV *UBC* genes have both C- and N-terminal extensions (22).

The fungal genome is composed of chromosomes, extrachromosomal copies of chromosomal genes, eg nucleosomes, and occasionally stable extrachromosomal elements. These extrachromosomal elements have developed a benignly parasitic relationship with their host, where they successfully balance the theft of cellular resource for the replication and segregation of the element, while not compromising the fitness of the host.

General reviews of fungal extrachromosomal elements are covered by references 5 and 6, while the DNA plasmids of the yeasts *Saccharomyces* species are covered by references 7 and 8 and *Kluyveromyces* species are covered by reference 9.

5

The 2 μ m plasmids of *Saccharomyces* species are extrachromosomal DNA species which have evolved mechanisms to ensure their long term autonomous survival without any associated phenotype. The 2 μ m plasmid resides in the nucleus and is packaged into chromatin. The plasmid origin of replication acts as an autonomously replicating sequence, while other sequences ensure the maintenance of a controlled high copy number and allow the plasmid to partition uniformly into the daughter cells at mitosis.

The plasmid is not required for normal mitotic growth and does not provide the host with any selective advantage since *Saccharomyces* species devoid of 2 μ m plasmid, denoted as cir⁰, grow only slightly faster than their 2 μ m plasmid containing, or cir⁺, parents.

The 2 μ m plasmid is a double stranded circular plasmid of approximately 6,318 bp, comprising two unique regions of 2,774 and 2,346 bp separated by a pair of exact inverted repeats, each 599 bp long (10). *In vivo* the monomeric plasmid exists as an equal mixture of the two inversion isomers (A and B) that form following site specific recombination between the two inverted repeats. The 2 μ m plasmid has four open reading frames known as *FLP* (also known as A), *REP1* (also known as B), *REP2* (also known as C) and *RAF* (also known as D). The plasmid also contains a region, located between *RAF* and the origin of replication, called *STB* or *REP3*, which is composed of a series of imperfect 62 bp repeat elements. This element is required in *cis*, along with the *trans* acting elements,

REP1 and *REP2*, to enable efficient partitioning of the plasmid between the mother and the daughter cell.

The 2 μ m plasmid copy number is also indirectly under the control of chromosomal genes, since it is known that 2 μ m plasmid copy number does vary between different *Saccharomyces cerevisiae* strains and because the chromosomal recessive mutation, known as *nib1*, results in clonal lethality due to uncontrolled amplification of 2 μ m plasmid copy number (39). Yeast strains carrying the *nib1* mutation resemble engineered yeast strains where FLP gene expression is galactose induced. The involvement of proteins of the fungal ATP-dependent ubiquitin protein degradation pathway in the regulation of fungal plasmid copy number is not described in the art. Nor is it disclosed that genes of the fungal ATP-dependent ubiquitin protein degradation pathway can be manipulated to control fungal plasmid copy number.

Although the 2 μ m plasmid is a very common genetic component of *Saccharomyces cerevisiae*, other yeast strains are known to contain identifiable DNA plasmids, notably the pSR1 and pSB3 plasmids (6,251 bp and 6,615 bp) of *Zygosaccharomyces rouxii*, the pSB1 and pSB2 plasmids (6,550 bp and 5,415 bp) of *Zygosaccharomyces bailii*, the pSM1 plasmid (5,416) of *Zygosaccharomyces fermentati* and the pKD1 plasmid (4,757 bp) of *Kluyveromyces drosophilarum* (9). The most striking feature of all these plasmids is their resemblance to the *Saccharomyces cerevisiae* 2 μ m plasmid. Each plasmid is circular, double stranded DNA and is composed of two approximately equally sized halves separated by inverted repeat sequences. Each plasmid contains a single Autonomously Replicating Sequence (ARS) close to one of the inverted repeat sequences

and three or four open reading frames, one of which encodes a recombinase which catalyses recombination between the inverted repeats.

A *Saccharomyces cerevisiae* plasmid is considered to be "2 μ m-based" if it
5 contains at least one of the 2 μ m plasmid elements (ARS, inverted repeat sequences or 2 μ m open reading frames), especially the ARS.

Summary of the Invention

10 One aspect of the present invention provides a process of producing a fungal cell derived product, comprising (i) providing a fungal cell having a plasmid, the plasmid comprising a functional coding sequence for a protein, and the fungal cell having a modified level of Ubc4p or Ubc5p (hereinafter, generically known as Ubcp activity), and (ii) culturing the
15 cell to produce the fungal cell derived product.

Preferably the fungal cell derived product is a desired protein encoded by the said coding sequence, and the said modified level of Ubcp activity is lower than normal for the cell. This can be tested *in vivo* by assaying for
20 the rate of abnormal protein turnover (3). The level of Ubcp (Ubc4p and/or Ubc5p) activity may be reduced to at most 50%, 40%, 30%, 20%, 10% or 1% of the wild-type level. Preferably, the cell has a minimal Ubc4p or Ubc5p activity. The cell should not, however, have a low level of both Ubc4p and Ubc5p, since its growth rate will generally be too low
25 to be useful.

The reduction in Ubcp activity can be achieved in any one of a variety of ways. Firstly the cell can produce a compound which interferes with the

binding of the *UBC*-encoded product to its receptor. Hence, a construct may be provided in the cell to express a polypeptide which competes for the binding of Ubc4p or Ubc5p to its target. This will facilitate a reduction in the effective Ubc4p or Ubc5p activity. This may be done by over-expressing the UBC domain encoded by *UBC4* or *UBC5* described above. It will be important to ensure that the over-expressed UBC domain encoded by *UBC4* or *UBC5* does not have any intrinsic Ubc4p or Ubc5p activity of its own, since this might actually contribute to the overall Ubc4p or Ubc5p activity. This may be achieved, by site directed mutagenesis, by removing or replacing (for example with an alanine) the cysteine which acts as the acceptor site for the ubiquitin within the UBC domain of *UBC4* (or 5) or other conserved amino acids within the UBC domain. Over expression of the inactive UBC domain of *UBC4* (or 5) may be achieved from its own endogenous promoter, or from any other convenient promoter. The construct may be integrated into the chromosome or episomal.

Alternatively, in order to achieve a reduced level of Ubcp activity, the endogenous *UBC* gene may be modified such that substantially no protein is produced therefrom or such that any protein produced therefrom has a reduced level of Ubc4p or Ubc5p activity. Thus, for example, the *UBC* gene may be deleted (either in a regulatory region or in the coding region or both) such that no polypeptide is produced or a mutant (defective) polypeptide product is produced. (By "regulatory region", we include parts of the genome acting on the *UBC* gene indirectly, for example a gene producing a *UBC* gene activator.) Deletion of all or part of the *UBC* open reading frame (14) is preferred, as this will reduce or abolish Ubcp activity and generate a non-reverting mutant fungal strain. Alternatively,

the activity can be reduced or abolished by classical mutagenesis procedures, whereby the DNA sequence of the *UBC* gene is mutated in such a way as to produce point mutations or deletions which modify and/or disrupt the normal amino acid sequence of the Ubcp. If a mutant
5 Ubcp polypeptide is produced, it may be unstable (ie be subject to increased protein turnover relative to the native protein); or unable to conjugate ubiquitin, or unable to deliver bound ubiquitin to its substrate.

For example, the *UBC* gene may be modified such that the ubiquitin-
10 accepting cysteine in any protein produced therefrom is absent or of reduced ubiquitin-accepting activity, for example due to alterations in the amino acid residues surrounding or otherwise interacting with the cysteine, as noted above in the context of producing competitive (but inactive) polypeptide. Alternatively, the *UBC* gene may be modified such
15 that the capacity of any mutant protein produced therefrom is unable to interact with or has reduced affinity for the E1 ubiquitin donor (product of the *UBA1* or *UBA2* genes). Alternatively, the *UBC* gene may be modified such that the capacity of any mutant protein produced therefrom to interact
20 with the final ubiquitin acceptor and/or the Ubiquitin ligase (E3) enzyme is absent or reduced. Specifically, mutations (deletion, insertions or substitutions) within the first 21 amino acids of the primary sequence and the first α helix (residues 3-13) of Ubc4p and Ubc5p (29) are preferred as the latter have been implicated in binding of Ubc2p, a related protein, to the ubiquitin protein ligase Ubr1p (33). Especially preferred are
25 mutations affecting the glutamic acid at position 10 (Glu-10) within the primary sequence of Ubc4p and Ubc5p, particularly replacement by lysine (Glu10Lys) or arginine (Glu10Arg).

Alternatively a different promoter may be used to control expression of the *UBC* gene; such a promoter may be regulatable. For example, it may be inducible, as are promoters of the galactose utilisation pathway, or derepressed by the removal of an inhibitor, as are promoters of the acid
5 phosphatase group.

Site-directed mutagenesis or other known techniques can be employed to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in reference 23. Suitable
10 mutations include chain termination mutations (clearly stop codons introduced near the 3' end might have insufficient effect on the gene product to be of benefit; the person skilled in the art will readily be able to create a mutation in, say, the 5' three quarters of the coding sequence), point mutations that alter the reading frame, small to large deletions of
15 coding sequence, mutations in the promoter or terminator that affect gene expression and mutations that de-stabilize the mRNA. Specific mutations can be introduced by an extension of the gene disruption technique known as gene transplacement (24).

20 Generally one uses a selectable marker to disrupt a gene sequence, but this need not be the case, particularly if one can detect the disruption event phenotypically. In many instances the insertion of the intervening sequence will be such that a stop codon is present in frame with the *UBC* sequence and the inserted coding sequence is not translated. Alternatively
25 the inserted sequence may be in a different reading frame to *UBC*.

A third principal way to achieve a reduction of Ubcp activity is for the cell to produce *UBC* antisense mRNA. This may be achieved in conventional

ways, by including in the cell an expression construct for an appropriate sequence. *UBC* antisense mRNA may be produced from a constitutive or regulated promoter system (eg promoters of the galactose catabolic pathway), thereby facilitating a reduction in translatable *UBC* mRNA.

- 5 Use of the regulated *UBC* antisense mRNA also allows for control of the ubiquitin-dependent protein degradation pathway by the addition or removal of the activator.

Fungal cells useful in the methods of the invention include the genera
10 *Pichia*, *Saccharomyces*, *Zygosaccharomyces*, *Kluyveromyces*, *Candida*,
Torulopsis, *Hansenula* (now reclassified as *Pichia*), *Schizosaccharomyces*,
Citeromyces, *Pachysolen*, *Debaromyces*, *Aspergillus*, *Metschnikowia*,
Rhodoporida, *Leucosporidium*, *Botryosphaeria*, *Endomyces*, *Trichoderma*,
Cephalosporium, *Hemicella*, *Mucor*, *Neurospora* and the like. Preferred
15 genera are *Pichia*, *Saccharomyces*, *Zygosaccharomyces* and
Kluyveromyces. Examples of *Saccharomyces* sp. are *Saccharomyces*
cerevisiae, *Saccharomyces italicus*, *Saccharomyces diastolicus* and
Zygosaccharomyces rouxii. Examples of *Kluyveromyces* sp. are
Kluyveromyces fragilis and *Kluyveromyces lactis*. Examples of *Hansenula*
20 sp. are *Hansenula polymorpha* (now *Pichia angusta*), *Hansenula anomala*
(now *Pichia anomala*) and *Pichia capsulata*. An example of a *Pichia* sp.
is *Pichia pastoris*. Examples of *Aspergillus* sp. are *Aspergillus niger* and
Aspergillus nidulans. *Yarrowia lipolytica* is an example of a suitable
Yarrowia species.

25

Preferred are yeast strains, and of these *Saccharomyces cerevisiae* is the particularly preferred host. The yeast strains used can be any haploid or diploid strain of *Saccharomyces cerevisiae*, but in the case of diploid

strains it is preferred that the activity of the Ubcp enzyme from both copies of the *UBC* gene is reduced or abolished.

A number of species have been shown to have homologues of
5 *Saccharomyces cerevisiae* *UBC4* and *UBC5* genes. *UBC4* and *UBC5*
homologues have been described in *Homo sapiens* (34), *Drosophila*
melanogaster (35), *Caenorhabditis elegans* (36), *Arabidopsis thaliana*
(37), *Schizosaccharomyces pombe* and *Candida albicans* (38). The
10 *Drosophila melanogaster* and *Caenorhabditis elegans* homologues, *UbcD1*
and *ubc-2*, respectively, have also been shown to have Ubcp activity. It
can be seen that a homologue need not be termed *UBC4* or *UBC5*;
equally, a gene which is called *UBC4* or *UBC5* need not be a homologue.

Of the known *UBC4/UBC5* homologues in the literature, the similarity of
15 the various proteins can be calculated by aligning the primary amino acid
sequences. A suitable program is the Megalign Program, Lasergene,
DNASTAR Inc, 1228 South Park Street, Madison, Wisconsin 53715,
USA. Using such a program the calculated percentage similarity ranges
from 75.7% to 97.3. These values are very high and reflect the highly
20 conserved nature of the Ubc proteins. The highly conserved cysteine
residue in the active site occurs at position 193 in the consensus sequence.

Of the other Ubc proteins, the calculated percentage similarity between
them and to *Saccharomyces cerevisiae* Ubc4p and Ubc5p ranged from
25 24.2% to 63.5%. Proteins homologous to the *Saccharomyces cerevisiae*
Ubc4p and Ubc5p can therefore be defined as any Class I (as defined by
Jentsch, 1992, reference 22) ubiquitin conjugating enzyme, which
possesses 66.7% or greater primary amino acid sequence similarity to

Saccharomyces cerevisiae Ubc4p or Ubc5p, as defined by the Megalign program. A gene is deemed to be homologous to *S. cerevisiae* *UBC4* or *UBC5* if it encodes such an enzyme.

- 5 A number of species have also been shown to possess Ubcp activity. As stated previously *ubc4/ubc5* double mutants of *Saccharomyces cerevisiae* have increased doubling time, reduced resistance to amino acid analogues and reduced resistance to heat shock. It is known that the *Drosophila* UBC1 protein, encoded by the *UbcD1* gene, which is 79.6% and 80.3% similar to *Saccharomyces cerevisiae* Ubc4p and Ubc5p respectively, can reverse the phenotypes of a yeast with no Ubc4p or Ubc5p activity when placed downstream of the *UBC4* promoter (35). Similarly it is also known that the *Caenorhabditis* protein *ubc-2*, encoded by the *ubc-2* gene, which is 78.2% and 78.9% similar to *Saccharomyces cerevisiae* Ubc4p and Ubc5p respectively, has the same properties (36). This is therefore a functional test of whether a protein from an unknown source has Ubc4p or Ubc5p activity. It can also be seen that, for the examples of doubling time and survival rate after 24hrs at 38°C, the single *ubc4* or *ubc5* mutant strains described by Seufert and Jentsch (3,36) have similar characteristics to the wild-type strain. The Ubcp activity of an unknown Ubc protein, or a mutant form of a known Ubc protein, relative to the natural *Saccharomyces cerevisiae* Ubc4p or Ubc5p, can be determined by its relative ability to return the doubling time or survival rate after 24hrs at 38°C (as described in references 3 or 36), of a double *ubc4/ubc5* mutant strain to normal for a wild type or single *ubc4* or *ubc5* mutant *Saccharomyces cerevisiae* strain once the unknown or mutant Ubc protein has been integrated into the *Saccharomyces cerevisiae* genome under the control of the endogenous *UBC4* or *UBC5* promoter, preferably as a

single copy integration at the endogenous *UBC4* or *UBC5* locus by procedures already described in the literature (36).

In a preferred aspect of the invention, the level of Ubc4p or Ubc5p activity is reduced. This has been found to increase the copy number of an expression plasmid in the cell, and to cause an increased level of expression of a desired protein expressed from the plasmid. Conversely, increasing the level of Ubc4p or Ubc5p activity will reduce the level of expression of the protein, which may be desirable in some circumstances, for example where the plasmid-encoded protein inhibits production of the desired protein.

The term "desired protein" is used herein in the normal sense to mean any protein (or other polypeptide) which is desired in a given process at a higher level than the one at which the fungal cell would, without human intervention, produce it. The desired protein may be endogenous to the species in question, for example it may be an enzyme which is normally produced by the host cell. Usually, however, the protein is heterologous to the host cell. The protein may perform its required task in the host cell or host cell culture without being extracted. Usually, however, the protein is extracted from the cell culture and purified to some extent for use elsewhere. The protein may be a viral, microbial, fungal, plant or animal protein, for example a mammalian protein. Preferably, it is a human protein, for example albumin, immunoglobulin or a fragment thereof (such as an Fab fragment or single chain antibody), (haemo-)globin, blood clotting factors (such as factors II, VII, VIII, IX), interferons, interleukins, α_1 -antitrypsin, insulin, calcitonin, cell surface receptors, fibronectin, pro-urokinase, (pre-pro)-chymosin, antigens for

vaccines, t-PA, tumour necrosis factor, erythropoietin, G-CSF, GM-CSF growth hormone, platelet-derived endothelial cell growth factor, and enzymes generally, such as glucose oxidase and superoxide dismutase. The protein is, of course, not Ubc4p or Ubc5p itself, nor a fusion of either
5 Ubc4p or Ubc5p in which the Ubc4p or Ubc5p performs its natural function.

The desired protein, if it is to be purified from the fungal cell culture, may be obtained by any technique suited to that protein. For example, albumin
10 may be purified from a *Saccharomyces*, *Kluyveromyces* or *Pichia* cell culture according to the techniques disclosed in WO96/37515, EP-625 202 or EP-464 590, respectively.

Our work has principally involved human albumin, although there is no
15 reason to suppose that the process of the invention is applicable only to this protein, especially since the invention has also been shown to be advantageous in the expression of human haemoglobin.

The term "human albumin" is used herein to denote material which is
20 indistinguishable from human serum albumin or which is a variant or fragment thereof. By "variant" we include insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the oncotic, useful ligand-binding or immunogenic properties of albumin. For example we include naturally-
25 occurring polymorphic variants of human albumin or human albumin analogues disclosed in EP-A-322 094. Generally, variants or fragments of human albumin will have at least 50% (preferably at least 80%, 90% or 95%) of human serum albumin's ligand binding activity (for example

bilirubin-binding) and at least 50% (preferably at least 80%, 90% or 95%) of human serum albumin's oncotic activity, weight for weight.

The desired protein coding region is preferably contained within a hybrid
5 plasmid comprising a promoter sequence, a DNA coding sequence which is under the transcriptional control of the promoter, a leader sequence directing the secretion of the protein and a DNA sequence containing a eukaryotic transcription termination signal, which plasmid is then maintained as an extrachromosomal DNA sequence or is integrated into
10 one or more chromosomes of the host organism.

Suitable promoters for the expression of the protein include those associated with the phosphoglycerate kinase (*PGK1*) gene, galactokinase (*GAL1*) and uridine diphosphoglucose 4-epimerase (*GAL10*) genes, iso-1-
15 cytochrome c (*CYC1*), acid phosphatase (*PHO5*), alcohol dehydrogenase genes (*ADH1* and *ADH2*) and MF α -1. The preferred promoters are the glycerol-3-phosphate dehydrogenase (*GPD1*), described in EP 424 117, and the protease B (*PRB1*) promoter, described in EP-431 880 B1.

20 Suitable transcription termination sequences can be the 3' flanking sequence of the eukaryotic gene which contains proper signals for transcription termination and polyadenylation in the fungal host, or those of the gene naturally linked to the expression control sequence, or those associated with the phosphoglycerate kinase (*PGK1*) or the iso-1-
25 cytochrome c (*CYC1*) gene. The preferred transcription termination sequence is from the alcohol dehydrogenase gene (*ADH1*).

- Suitable secretory leader sequences are, for example, the natural human serum albumin leader sequence, the leader sequence from the *Saccharomyces cerevisiae* MF α -1 leader sequence, the *Kluyveromyces lactis* killer toxin leader, a fusion between the natural human serum albumin leader and the *Saccharomyces cerevisiae* MF α -1 leader sequence, or a fusion between the *Kluyveromyces lactis* killer toxin leader and the *Saccharomyces cerevisiae* MF α -1 leader sequence, or conservatively modified variations of these sequences, as described in WO 90/01063.
- Hybrid plasmids may also be used which, apart from the expression control sequence, the heterologous gene sequence and the transcription termination sequence, contain additional sequences which are non-essential or less important for the function of the promoter, ie for the expression of the desired polypeptide, but which perform important functions in, for example, the propagation of the cells transformed with the said hybrid plasmids. The additional DNA sequences may be derived from prokaryotic and/or eukaryotic cells and may include chromosomal and/or extra-chromosomal DNA sequences. For example, the additional DNA sequences may stem from (or consist of) plasmid DNA, such as bacterial, yeast or higher eukaryotic chromosomal DNA. Preferred hybrid plasmids contain additional DNA sequences derived from bacterial plasmids, especially *Escherichia coli* plasmid pBR322 or related plasmids, bacteriophage, yeast 2 μ m plasmid, and/or yeast chromosomal DNA.
- In the preferred hybrid plasmids for the expression of the heterologous polypeptide, the additional DNA sequences carry a yeast replication origin and a selective genetic marker for yeast. Hybrid plasmids containing a yeast replication origin, eg an autonomously replicating segment (ARS),

are extrachromosomally maintained with the yeast cells after transformation and are autonomously replicated upon mitosis. Hybrid plasmids containing sequences homologous to the yeast 2 μ m plasmid DNA can be as well. These hybrid plasmids may be integrated by recombination into yeast 2 μ m plasmids already present within the cell or may replicate autonomously. The integration vectors of EP-A-251 744 or the "disintegration" vectors of EP-A-286 424 may be used.

Advantageously, the additional DNA sequences which are present in the hybrid plasmids also include a replication origin and a selective marker for the bacterial host, especially *Escherichia coli*, and a selectable marker for the final fungal host. There are useful features which are associated with the presence of an *Escherichia coli* replication origin and an *Escherichia coli* marker in a yeast hybrid plasmid. Firstly, large amounts of hybrid plasmid DNA can be obtained by growth and amplification in *Escherichia coli* and, secondly, the construction of hybrid plasmids is conveniently done in *Escherichia coli* making use of the whole repertoire of cloning technology based on *Escherichia coli*. *Escherichia coli* plasmids, such as pBR322 and the like, contain both *Escherichia coli* replication origin and *Escherichia coli* genetic markers conferring resistance to antibiotics, for example tetracycline and ampicillin, and are advantageously employed as part of the yeast hybrid vectors. The selective fungal marker may be any gene which facilitates the selection of transformants due to the phenotypic expression of the marker. Suitable markers are particularly those expressing antibiotic resistance or, as in the case of auxotrophic yeast mutants, genes which complement host lesions. Corresponding genes confer, for example, resistance to the antibiotic cycloheximide or provide

for prototrophy in an auxotrophic yeast mutant, for example the *URA1*, *URA3*, *LEU2*, *HIS3*, *HIS4*, *TRP5*, *TRP1* and *LYS2* genes.

It has been demonstrated that fungal cells of the genera *Pichia*,
5 *Saccharomyces*, *Kluyveromyces*, *Yarrowia* and *Hansenula* can be transformed by enzymatic digestion of the cell walls to give spheroplasts; the spheroplasts are then mixed with the transforming DNA and incubated in the presence of calcium ions and polyethylene glycol, then transformed spheroplasts are regenerated in regeneration medium. The regeneration
10 medium is prepared in such a way as to allow regeneration and selection of the transformed cells at the same time.

Since the yeast genes coding for enzymes of nucleic acid or amino acid biosynthetic pathways are generally used as selection markers, the
15 regeneration is preferably performed in yeast minimal medium. Methods for the transformation of *Saccharomyces cerevisiae* are taught generally in EP 251 744, EP 258 067, WO 90/01063 and by Hinnen *et al* (4), all of which are incorporated herein by reference.

20 Hence, in its broadest aspect, the invention provides the use of a means to vary *UBC4* or *UBC5* function in a fungal cell to control the copy number of a plasmid in that cell.

Preferred non-limiting embodiments of the invention will now be
25 described by way of example and with reference to the accompanying drawings, in which:

Figure 1 is a plasmid map of pDB2277;

Figure 2 is photograph of a rocket immunoelectrophoresis gel showing the increased rHA productivity of *ubc5* disrupted yeast. Strains were as follows: Sample 1, DS569 *ura3* [pAYE329/YCplac33]; Samples 2-17, DS569 *ura3* [pAYE329/pDB2276] transformants 1-16; Samples 18-33, DS1101 *ura3* [pAYE329/pDB2276] transformants 1-16; Samples 34-38 HSA standards 100, 75, 50, 30, and 20 µg/mL HSA;

Figure 3 is the genomic DNA sequence of the yeast *Saccharomyces cerevisiae* gene *UBC4*; the 2.066 kb sequence extends from the *Pst*I site 0.95 kb upstream of the start of the *UBC4* open reading frame to the *Pst*I site 0.58 kb downstream of the translation stop codon;

Figure 4 is a plasmid map of pAYE399;

Figure 5 is a plasmid map of pAYE400;

Figure 6 is a plasmid map of pUBT1;

Figure 7 is a plasmid map of pUBT2;

Figure 8 is a plasmid map of pHbD2-1;

Figure 9 is a plasmid map of pAYE792;

Figure 10 is a plasmid map of pBST+;

Figure 11 is a plasmid map of pDB2258;

Figure 12 is a plasmid map of pDB2259;

Figure 13 is a plasmid map of pDB2260;

Figure 14 is a plasmid map of pDB2261;

Figure 15 is the genomic DNA sequence of the *Saccharomyces cerevisiae* *UBC5* gene; the 1.2 kb sequence extends from the *Bgl*II site 0.55 kb upstream of the start of the *UBC5* open reading frame to the *Bcl*II site 0.12 kb downstream of the translation stop codon;

Figure 16 is a plasmid map of pDB2262;

Figure 17 is a plasmid map of pDB2264;

Figure 18 is a plasmid map of pDB2275; and

Figure 19 is a plasmid map of pDB2276.

Detailed description of the invention

- 5 All standard recombinant DNA procedures are as described in reference 13 unless otherwise stated.

Example 1: Disruption of the *Saccharomyces cerevisiae* *UBC4* gene

- 10 The *Saccharomyces cerevisiae* *UBC4* gene is located on chromosome II. The DNA sequence of the *UBC4* gene is shown in Figure 3.

- The *UBC4* gene was mutated by the process of gene disruption (14) which deleted the entire *UBC4* open reading frame, thereby preventing
15 production of active Ubc4 protein. This was achieved by first amplifying by PCR a suitable marker gene (*URA3*) with mutagenic single stranded DNA primers which modified the 5' and 3' ends of the *URA3* gene so as to include DNA sequences identical to regions 5' and 3' to the *UBC4* open reading frame and then transforming a *ura3* auxotrophic yeast strain to
20 uracil prototrophy.

- Two single stranded oligonucleotide primers (*UBC4URA1* and *UBC4URA2*) suitable for PCR amplification of the 5' and 3' ends of the *URA3* gene, incorporating *UBC4* sequences at the extremes, were
25 synthesised using an ABI 380B DNA Synthesiser.

- UBC4URA1 5'TTTCATCGTC CAATCCCATA TAAATCTTGC
TTCTCTTTTT CAGCTGAGTA AGCTTTTCAA
TTC ATCTTTT-3'
- 5 UBC4URA2 5'-TCTTATTTTT CATCTTAATA AATAATCCAG
AGAATAAATC TATCCTGAAA AGCTTTTTTCT
TTCCAATTTT-3'

PCR reactions were performed to amplify the *URA3* gene from the
10 plasmid YEp24 (15). Conditions were as follows: 1µg/mL plasmid
YEp24 DNA, 2µM of each primer, denature at 94°C for 30 seconds,
anneal to 45°C for 40 seconds, extend at 72°C for 120 seconds for 20
cycles, followed by a 72°C soak for 600 seconds, followed by a 4°C soak,
using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus
15 PCR kit employing AmpliTaq Thermal Stable DNA Polymerase, total
reaction volume 50µL, according to the manufacturer's instructions.
Alternative conditions were, 2ng/mL plasmid YEp24 DNA, 0.1µM of
each primer, denature at 94°C for 30 seconds, anneal to 55°C for 40
seconds, extend at 72°C for 120 seconds for 30 cycles, followed by a 72°C
20 soak for 600 seconds, followed by a 4°C soak, using a Perkin-Elmer-Cetus
Thermal Cycler and a Perkin-Elmer-Cetus PCR kit employing AmpliTaq
Thermal Stable DNA Polymerase, total reaction volume 50µL, according
to the manufacturer's instructions. The product, 5'-*UBC4-URA3-UBC4*-
3', was analysed by gel electrophoresis and was found to be of the
25 expected size, approximately 1.2kb. The amplified PCR products were
purified using a Promega Wizard PCR DNA purification kit according to
the manufacturer's instructions.

The *Saccharomyces cerevisiae* strain DS569 *cir*⁰ (16) was transformed to leucine prototrophy with the recombinant human albumin (rHA) secretion plasmid pAYE329 (19). The promoter sequence in this plasmid corresponds to that of the *Saccharomyces cerevisiae* NAD-linked glycerol-3-phosphate dehydrogenase (*GPD1*) gene, rather than the FAD-linked glycerol-3-phosphate dehydrogenase (*GUT2*) gene as originally described (19).

The *ura3* auxotrophic derivative of the *Saccharomyces cerevisiae* strain DS569 [pAYE329] was created by mutating the *URA3* gene by the process of gene disruption (14) which deleted part of the *URA3* coding sequence, thereby preventing the production of active Ura3 protein. The plasmid YEp24 (15) was digested to completion with *Hind*III and the products resolved by gel electrophoresis. The 1.17kb *Hind*III *URA3* gene fragment was isolated and ligated into the unique *Hind*III site of pACYC184 (17) to create plasmid pAYE399, Figure 4. Plasmid pAYE399 was digested to completion with *Pst*I and partially digested with *Nco*I, the products were resolved by gel electrophoresis and the 5.41kb *Nco*I-*Pst*I DNA fragment lacking the central part of the *URA3* gene was isolated, blunt-end filled with the Klenow fragment of DNA Polymerase and religated. The resultant plasmid pAYE400, Figure 5, possesses a deletion within the *URA3* open reading frame and an *Nco*I site at the deletion site. The deletion derivative of *URA3* gene (Δ *URA3*) was isolated as a 0.94kb *Hind*III fragment from plasmid pAYE400. A *ura3* auxotrophic mutant of DS569 [pAYE329] was created by transforming DS569 [pAYE329] with the Δ *URA3* 0.94kb *Hind*III fragment and selecting for Ura⁻ yeast by resistance to 5-fluoro-orotic acid (18). Colonies able to grow on this medium were purified, tested to verify that they were unable to grow in

the absence of uracil supplementation and that the defect could be complemented by introduction of the *URA3* gene by transformation.

One such strain, DS569 *ura3* [pAYE329], was transformed to uracil prototrophy with the 5'-*UBC4-URA3-UBC4*-3' PCR product. A Southern blot of digested genomic DNA of a number transformants was probed with the *UBC4* gene as a 2.07kb *Pst*I DNA fragment and confirmed the disruption of the *UBC4* gene. The new strain was designated UB05 [pAYE329].

10

These methods are equally applicable to the disruption of *UBC4* in any haploid *Saccharomyces cerevisiae* strain. If the desired host already carries a *ura3* auxotrophic mutation, then disruption of *UBC4* can be performed with the 5'-*UBC4-URA3-UBC4*-3' PCR product described above. If the desired haploid host does not carry a *ura3* auxotrophic mutation, then disruption of *UBC4* can be performed once the strain has been made *ura3* by transformation with the Δ *URA3* 0.94kb *Hind*III fragment from pAYE400 and selecting for Ura⁺ yeast by resistance to 5-fluoro-orotic acid as described above. In the case of a diploid host it is necessary to disrupt both *UBC4* genes. This can be achieved by disrupting the *UBC4* gene in each of the two parental haploid strains first before diploidisation.

15

20

Example 2: Disruption of the *Saccharomyces cerevisiae* *UBC4* gene enhanced the production of recombinant human albumin.

25

The rHA productivity of the yeast strain DS569 [pAYE329] (which does not have a *UBC4* disruption) and two independent isolates of UB05

[pAYE329], called UB05-1 [pAYE329] and UB05-6 [pAYE329] (both of which do have a *UBC4* disruption) was assessed in 10mL shake flask culture. Yeast were inoculated into YNB (Difco) minimal medium, buffered with sodium phosphate/citrate pH 6.0 and containing 2% w/v glucose, and incubated at 30°C, 200 rpm for 3 days. The rHA productivity was estimated by rocket immunoelectrophoresis against HSA standards (25-150µg/mL). The rHA productivity of DS569 [pAYE329] under these conditions was calculated to be 45mg/L, while the rHA productivity of the two UB05 [pAYE329] isolates measured under identical conditions was calculated to be 77 and 75 mg/L, respectively.

Example 3: Disruption of the *Saccharomyces cerevisiae* *UBC4* gene increases hybrid 2µm plasmid copy number.

The plasmid copy number of the hybrid 2µm plasmid of the yeast strains DS569 [pAYE329] and two independent isolates of UB05 [pAYE329], called UB05-1 [pAYE329] and UB05-6 [pAYE329], was assessed in 100mL shake flask culture. Yeast were inoculated into YNB minimal medium, buffered with sodium phosphate/citrate pH 6.0 and containing 2% w/v glucose, and incubated at 30°C, 200 rpm for sufficient time, usually 1 to 2 days, to allow the culture density to exceed 5 AU/mL, equivalent to mid-logarithmic growth phase. Total genomic DNA was extracted by glass disruption of the yeast cells, followed by solvent extraction, dialysis and ethanol precipitation. The total genomic DNA was digested to completion with *HindIII* and the products analysed by gel electrophoresis. The ethidium bromide staining of the plasmid specific DNA bands increased relative to the ethidium bromide staining of the ribosomal DNA (rDNA) bands, indicating that the plasmid copy number

of the hybrid 2 μ m plasmid had increased. Quantitation of the hybrid 2 μ m plasmid copy number relative to the copy number of the rDNA was performed by Southern blot analysis with a joint rDNA/HSA cDNA probe. This showed that the plasmid copy number of the hybrid 2 μ m plasmid pAYE329 increased from 48.9 ± 9.2 copies per haploid genome in DS569 [pAYE329] to 83.1 ± 12.5 and 116.8 ± 29.0 copies per haploid genome in UB05-1 [pAYE329] and UB05-6 [pAYE329], respectively.

Example 4: Antisense *UBC4* mRNA expression.

10

One way to disrupt expression of the *UBC4* gene is to arrange for expression of an antisense polynucleotide.

15

The antisense transcript can be expressed from a copy, or copies, of the antisense expression cassette which have been integrated into the chromosome(s), or it can be expressed from a low plasmid copy number vector, eg a centromeric vector like YCp50 (25) or YCplac1111, YCplac33, YCplac22 (26) or plasmids p413 through to p416 containing the *GALI*, *GALL* or *GALS* promoters (27). The antisense transcript can also be expressed from a high plasmid copy number vector like pJDB207 (12), YEp13 or YEp24 (15). All of these expression plasmids or integrating cassettes require a yeast selectable marker eg *URA3*, *HIS3* or *TRP1* to facilitate selection during transformation of yeast containing the appropriate auxotrophic marker(s).

25

The promoter used to drive the expression of the antisense *UBC4* or antisense *UBC5*, may be the native promoter, or a related promoter. This has the advantage of promoting expression of the antisense transcript at the

same time as the appearance of the sense transcript. In an especially preferred embodiment, the antisense expression cassette is provided on a high plasmid copy number plasmid to ensure an excess of the antisense transcript over the sense transcript. Alternative promoters include strong
5 constitutive promoters such as the glycolytic promoters, eg *PGK1*, *PYK1*, *TDH2/TDH3* and *ENO1/ENO2*. Use of strong regulated promoters will have the advantage that plasmid copy number can be regulated at the will of the operator. Examples of such promoters are the *GAL1*, *GALL* and *GALS* promoters (Mumberg *et al*, 27). These galactose-induced
10 promoters have been incorporated into both high and low plasmid copy number vectors, separated from the *CYC1* terminator by a multiple cloning site. The example described below utilises a plasmid called p426 *GAL1* (Mumberg *et al*, 27). The antisense *UBC4* transcript can be effective in inactivating *UBC4* sense transcript only if the host fungal
15 strain contains a proficient *UBC4* gene. However, expression of a *UBC4* antisense transcript in a *ubc4* fungal strain may be beneficial in mopping up other *UBC4*-like transcripts, so this is an option as well.

Two single stranded oligonucleotide primers (*UBC43* and *UBC44*) suitable
20 for PCR amplification of the *UBC4* open reading frame were synthesised using an ABI 380B DNA Synthesiser.

UBC43 5'-ATAACAAGC TTCCAAAAAA ACATGATTTC ACT
 GACTATA GAGTACATAC-3'

25

UBC44 5'-GTAAGGACTT AAGCTTTATA CAGCGTATTT CT
 TTGTCCAT TCTCTGGCTG TAGC-3'

PCR reactions were performed to amplify the *UBC4* gene from genomic DNA prepared from the yeast strain S288C. Conditions were as follows: 5µg/mL S288C genomic DNA, 2µM of each primer, denature at 94°C for 30 seconds, anneal to 45°C for 40 seconds, extend at 72°C for 120 seconds for 35 cycles, followed by a 72°C soak for 600 seconds, followed by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer, Cetus PCR kit employing AmpliTaq Thermal Stable DNA Polymerase, total reaction volume 50µL, according to the manufacturer's instructions. The product, 5'-(*Hind*III)-*UBC4*-(*Hind*III)-3'; was analysed by gel electrophoresis and was found to be of the expected size, approximately 0.58kb. The amplified PCR products were purified using a Promega Wizard PCR DNA purification kit according to the manufacturer's instructions. Use of these two primers, UBC43 and UBC44, introduced *Hind*III sites 5' and 3' to the *UBC4* open reading frame.

15

The purified PCR product, 5'-(*Hind*III)-*UBC4*-(*Hind*III)-3', was digested to completion with *Hind*III, and ligated into the unique *Hind*III site situated between the *GAL1* promoter and the *CYC1* terminator of plasmid p426GAL1 (Mumberg *et al*, 27) generating two plasmids pUBT1 and pUBT2 (Figures 6 and 7). Plasmid pUBT1 contained the *UBC4* open reading frame so orientated as to produce an antisense *UBC4* transcript from the *GAL1* promoter, while plasmid pUBT2 contained the *UBC4* open reading frame so orientated as to produce a sense *UBC4* transcript from the *GAL1* promoter.

25

Yeast strains deficient in uracil biosynthesis due to the presence of a non-functional *ura3* gene, such as DS569 *ura3* [pAYE329] (Example 1), were transformed to uracil prototrophy with plasmid pUBT1. *UBC4* antisense

transcript production was induced by switching from a yeast growth medium containing glucose as the sole carbon source to a medium containing galactose as the sole carbon source. Conversely, *UBC4* anti-sense transcript production was repressed by switching from a yeast growth medium containing galactose as the sole carbon source to a medium containing glucose as the sole carbon source.

Example 5: Sense *UBC4* mRNA expression from the *GALI* promoter

Plasmid pUBT2 (Figure 7) allows for the over-expression of the *UBC4* transcript. In a *ubc4* deficient fungal strain transformed with plasmid pUBT2, when the carbon source is switched from glucose to galactose, *UBC4* mRNA expression will be increased and will force plasmid copy number down. This is yet another way to facilitate control over plasmid copy number by switching between repressing and activating carbon sources. Again this can be done in either a *ubc4* or *UBC4* background.

Yeast strains deficient in uracil biosynthesis due to the presence of a non-functional *ura3* gene, such as DS569 *ura3* [pAYE329] (16) or a *ura3* derivative of UB05 [pAYE329] (Example 1), were transformed to uracil prototrophy with plasmid pUBT2. *UBC4* sense transcript production was induced by switching from a yeast growth medium containing glucose as the sole carbon source to a medium containing galactose as the sole carbon source. Conversely, *UBC4* sense transcript production from pUBT2 was repressed by switching from yeast growth medium containing galactose as the sole carbon source to a medium containing glucose as the sole carbon source.

Example 6: Disruption of the *Saccharomyces cerevisiae* *UBC4* genes enhances the production of other recombinant human proteins.

5 Elimination of the *ubc4* gene will increase the expression of other heterologous proteins. This was exemplified by analysing the expression level of recombinant human haemoglobin in DS569 and DS1101 (described later in Example 7) which possesses a mutation within the *UBC4* open reading frame. The human haemoglobin expression plasmid,
10 called pHbD2-1 (Figure 8), was based on the whole 2 μ m disintegration vector pSAC35 (16). Transcription of the human α -globin chain was directed by the *GPD1* promoter (19) and terminated by the *PGKI* terminator. Transcription of the human β -globin chain was directed from the *PRB1* promoter and terminated by the *ADHI* terminator (16).

15

The rHb productivity of the yeast strains DS569 and DS1101 transformed to leucine prototrophy with pHbD2-1 was assessed in 10mL shake flask culture. Yeast were inoculated into YNB minimal medium, buffered with sodium phosphate/citrate pH 6.0 and containing 2% (w/v) glucose, and
20 incubated at 30°C, 200 rpm for 3 days. The rHb productivity in yeast soluble cell extracts was quantitated by a spectrophotometric assay from the height of the Soret peak in a second derivative spectrum, by comparison with standard HbA of known concentration (28). Total soluble protein concentration was quantitated by Coomassie Protein Assay
25 Reagent, according to the Manufacturer's Instructions (Pierce). The expression level of soluble rHb in DS569 [pHbD2-1] was calculated to be equivalent to 0.4% (w/v) total soluble protein. The expression level of

soluble rHb increased to 0.8% (w/v) in the strain DS1101 [pHbD2-1] carrying the *ubc4* deletion.

Example 7: Mutation of the *Saccharomyces cerevisiae* *UBC4* gene

5 As described above, the original mutation was produced by random chemical mutagenesis. The starting strain for this process was DS569 [pAYE329] (16). DS569 [pAYE329] was subjected to chemical mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and
10 potential rHA over-expressing mutant strains selected by a plate screening procedure described in EP431880. One such mutant strain was called DS1101 [pAYE329]. Analysis of the rHA productivity of DS569 [pAYE329] and DS1101 [pAYE329] was performed in 10mL shake flask culture as described in Example 2. The rHA productivity was estimated by
15 rocket immunoelectrophoresis against HSA standards (25-150µg/mL). The rHA productivity of DS569 [pAYE329] under these conditions was calculated to be approximately 45mg/L, while the rHA productivity of DS1101 [pAYE329], measured under identical conditions, was calculated to be 78mg/L.

20 The plasmid copy number of the hybrid 2µm plasmid of the yeast strains DS569 [pAYE329] and DS1101 [pAYE329] was assessed in 100mL shake flask culture, as described in Example 3. Quantitation of the hybrid 2µm plasmid copy number relative to the copy number of the rDNA was
25 performed by Southern blot analysis with a joint rDNA/HSA cDNA probe. This showed that the plasmid copy number of the hybrid 2µm plasmid pAYE329 increased from 48.9 ± 9.2 copies per haploid genome

in DS569 [pAYE329] to 70.5 ± 15.9 copies per haploid genome in DS1101 [pAYE329].

To enable the identification of the nature of the original mutation which
5 was responsible for the increased plasmid copy number and rHA
productivity observed in DS1101 [pAYE329] a partial *Sau3A* genomic
DNA library was prepared from DS569 high molecular weight genomic
DNA in the centromeric vector YCp50 (30). A new yeast strain DS1101
ura3 [pAYE329] was prepared from DS1101 [pAYE329] by the method
10 described in Example 1. DS1101 *ura3* [pAYE329] was transformed to
uracil prototrophy with DNA from the DS569 YCp50 genomic library.
The transformants were assayed for reduced rHA expression by an anti-
HSA antibody dependant plate screening procedure described in
EP431880. One isolate, DS1101 *ura3* [pAYE329/pAYE792], was
15 identified which had reduced rHA productivity when assessed in 10mL in
shake flask culture. Yeast were inoculated into YNB (Difco) minimal
medium, buffered with sodium phosphate/citrate pH 6.0 and containing
2% w/v glucose, and incubated at 30°C, 200 rpm for 3 days. The rHA
productivity was estimated by rocket immunoelectrophoresis against HSA
20 standards and was shown to be reduced compared to the DS1101 *ura3*
[pAYE329/YCp50] control, but similar to the DS569 *ura3*
[pAYE329/YCp50] control. The plasmid copy number of the hybrid 2 μ m
plasmid of the yeast strains DS569 *ura3* [pAYE329/YCp50], DS569 *ura3*
[pAYE329/pAYE792], DS1101 *ura3* [pAYE329/YCp50] and DS1101
25 *ura3* [pAYE329/pAYE792] was assessed in 100mL shake flask culture, as
described in Example 3. Quantitation of the hybrid 2 μ m plasmid copy
number relative to the copy number of the rDNA was performed by
Southern blot analysis with a joint rDNA/HSA cDNA probe. This

showed that the plasmid copy number of the hybrid 2 μ m plasmid pAYE329 reduced from 59.4 ± 6.0 copies per haploid genome in DS1101 *ura3* [pAYE329/YCp50] to 38.3 ± 1.3 copies per haploid genome in DS1101 *ura3* [pAYE329/pAYE792], but remained unchanged in DS569 *ura3* [pAYE329/YCp50] and in DS569 *ura3* [pAYE329/pAYE792] at 33.0 ± 5.3 and 27.5 ± 4.5 copies per haploid genome, respectively.

The pAYE792 centromeric plasmid DNA was isolated from strain DS1101 *ura3* [pAYE329/pAYE792] (31) into *E.coli* and DNA sequenced (Figure 9). This revealed that the plasmid pAYE792 contained a contiguous 9.05kb genomic insert from chromosome II of *Saccharomyces cerevisiae* (32) spanning the region incorporating the *UBC4*, *TEC1* and *MIS1* genes. Subsequent subcloning of the three individual genes showed that the *UBC4* gene was responsible for the reduced rHA productivity and reduced plasmid copy number associated with pAYE792 in the strain DS1101 *ura3* [pAYE329/pAYE792].

In order to establish the nature of the mutation introduced into DS1101 by the NTG mutagenesis of DS569 the *UBC4* gene from DS1101 was isolated by PCR. Two single stranded oligonucleotide primers (UBC4A and UBC4B) suitable for the PCR amplification of the 2.1kb *UBC4* genomic *Pst*I fragment (Figure 3) were prepared using an ABI 380B DNA Synthesiser.

UBC4A 5'-ACTCCTGCAG TTATTCTTCT GCC-3'

UBC4B 5'-GTGTACAATA AGCTGCAGTA CTC-3'

PCR reactions were performed to amplify the *UBC4* gene from high molecular weight genomic DNA prepared from DS1101 according to reference 30. Conditions were as follows: 50ng/mL to 0.5ng/mL DS110 genomic DNA, 2 μ M of each primer, denature at 94°C for 30 seconds, anneal to 50°C for 40 seconds, extend at 72°C for 120 seconds for 40 cycles, followed by a 72°C soak for 600 seconds, followed by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit employing AmpliTaq Thermal Stable DNA Polymerase, total reaction volume 50 μ L, according to the manufacturer's instructions. The amplified 2.1kb DNA product was purified by TAE agarose gel electrophoresis by GeneClean III DNA extraction kit (Bio101 Inc., 1070 Joshua Way, Vista, CA 92083, USA) and digested to completion with *Pst*I. The plasmid pBST+ (Figure 10) was prepared from the phagemid pBS+ (Stratagene, 11011 North Torrey Pines Road, La Jolla, California 92037, USA) by digesting pBS+ with *Eco*RI and *Hind*III. The isolated linearised vector was ligated with a double stranded oligonucleotide linker with the sequence:

```

5' -
20  AGCTCCTAGGCCCCGGGCGGCCGCAAGCTTGTGCGACGCTAGCTGCAGAAGG
   3' -GGATCCGGGCCCCGCCGGCGTTTCGAACAGCTGCGATCGACGTCTTCC

ATCCAGATCTCGAGGCGCCATCGAT-3'
TAGGTCTAGAGCTCCGCGGTAGCTATTAA-5'

```

Plasmid pBST+ was linearised with *Pst*I and ligated with the *Pst*I digested PCR amplified 2.1kb *UBC4* DNA product to generate four separate plasmid isolates, called pDB2258, pDB2259, pDB2260 and pDB2261 (Figures 11-14). The *Pst*I inserts of all four plasmids (DS1101 derived) and the *UBC4* gene isolated from pAYE792 (DS569 derived) were DNA sequenced. The DNA sequence analysis revealed a mutation within the

DS1101 *UBC4* gene. This mutation, a G to an A substitution, was located in the tenth codon and had the DNA sequence:

5 DS569 *UBC4* gene: ATG TCT TCT TCT AAA CGT ATT GCT AAA GAA CTA
Met Ser Ser Ser Lys Arg Ile Ala Lys Glu Leu

DS1101 *UBC4* gene: ATG TCT TCT TCT AAA CGT ATT GCT AAA AAA CTA
Met Ser Ser Ser Lys Arg Ile Ala Lys Lys Leu

- 10 The mutation was such that it would change the tenth amino acid from a glutamic acid to a lysine, denoted as Glu10Lys.

This mutant form, or indeed any mutant form, of the *UBC4* gene can be introduced into any strain in which the *UBC4* gene has already been
15 disrupted by *URA3*, as already described in Example 1, by procedures similar to those already described in the literature for the replacement of the endogenous *Saccharomyces cerevisiae UBC4* gene by the *Caenorhabditis elegans ubc-2* gene (36). The yeast strain UB05 (Example 1) was transformed to *ura3* (Ura-) with the 2.1kb *Pst*I fragment from
20 either of the plasmids pDB2258, pDB2259, pDB2260 or pDB2261 (Figures 11-14) and selecting for Ura⁻ yeast by resistance to 5-fluoroorotic acid (18). Colonies able to grow on this medium were purified, tested to verify that they were unable to grow in the absence of uracil supplementation and that the defect could be complemented by
25 introduction of the *URA3* gene by transformation. Removal of the *URA3* gene from the *UBC4* locus in UB05 and its replacement by the Glu10Lys mutant form of the *UBC4* gene was confirmed by Southern Blot.

Example 8: Disruption of the *Saccharomyces cerevisiae* *UBC5* gene

The *Saccharomyces cerevisiae* *UBC5* gene is located on chromosome IV. The DNA sequence of the *UBC5* gene is shown in Figure 15.

5

The *UBC5* gene was mutated by the process of gene disruption (14) which deleted the entire *UBC5* open reading frame, thereby preventing production of active Ubc5 protein. This was achieved by first amplifying by PCR a suitable marker gene (*URA3*) with mutagenic single stranded
10 DNA primers which modified the 5' and 3' ends of the *URA3* gene so as to include DNA sequences identical to regions 5' and 3' to the *UBC5* open reading frame and then transforming a *ura3* auxotrophic yeast strain to uracil prototrophy.

15 Two single stranded oligonucleotide primers (*UBC5URA1* and *UBC5URA2*) suitable for PCR amplification of the 5' and 3' ends of the *URA3* gene, incorporating *UBC5* sequences at the extremes, were synthesised using an ABI 380B DNA Synthesiser.

20 *UBC5URA1* 5'-AGGACTGCTT ATTGACTACC ATCTTGAAAA
 GTCATTTTCT GCTCACCACC AGCTTTTCAA
 TTCATCTTTT-3'

UBC5URA2 5'-TTGATGTGTG CGCTGAGGAA GGTAAGTCTA
25 CACAATTTAT CCGTTAGCCC AGCTTTTTTCT
 TTCCAATTTT-3'

PCR reactions were performed to amplify the *URA3* gene from the plasmid YEp24 (15). Conditions were as follows: 1µg/mL plasmid YEp24 DNA, 2µM of each primer, denature at 94°C for 30 seconds, anneal to 45°C for 40 seconds, extend at 72°C for 120 seconds for 20 cycles, followed by a 72°C soak for 600 seconds, following by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit employing AmpliTaq Thermal Stable DNA Polymerase, total reaction volume 50µL, according to the manufacturer's instructions.

Alternative conditions were, 2ng/mL plasmid YEp24 DNA, 0.1µM of each primer, denature at 94°C for 30 seconds, anneal to 55°C for 40 seconds, extend at 72°C for 120 seconds for 30 cycles, followed by a 72°C soak for 600 seconds, followed by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit employing AmpliTaq Thermal Stable DNA Polymerase, total reaction volume 50µL, according to the manufacturer's instructions. The product, 5'-*UBC5-URA3-UBC5*-3', was analysed by gel electrophoresis and was found to be of the expected size, approximately 1.2kb. The amplified PCR product was purified using a Promega Wizard PCR DNA purification kit according to the manufacturer's instructions.

20

DS569 *ura3* [pAYE329] was transformed to uracil prototrophy with the 5'-*UBC5-URA3-UBC5*-3' PCR product. A Southern blot of digested genomic DNA of a number of transformants was probed with the *UBC5* gene as a 0.5kb *MluI*-*Asp718* DNA fragment and confirmed the disruption of the *UBC5* gene. The new strain was designated UB1 [pAYE329].

25

In an alternative method to disrupt the *UBC5* gene portions corresponding to the 5' and 3' ends of the *UBC5* gene were cloned by PCR. Two pairs

of single stranded oligonucleotide primers suitable for PCR amplification of the 5' end of the *UBC5* gene (DS101 and DS102) and the 3' end of the *UBC5* gene (DS103 and DS104), were synthesised using an ABI 380B DNA Synthesiser.

5

DS101 5'-TGACGCGGCC GCTCTAGATG TATTGCTAGT
GCTAGTACGG TG-3'

10

DS102 5'-TGACGTCGAC AAGCTTGGAA AATAAACTC
CAACCATC-3'

DS103 5'-TGACAAGCTT GTGTAGACTT ACCTTCCTCA
GCGC-3'

15

DS104 5'TGACGCTAGC ACGCGTCTGA CTTCTAATCA
GAAGATTATG GG-3'

20

PCR reactions were performed to amplify the 5' end of the *UBC5* gene. Conditions were as follows: 1000-10ng/mL S288C genomic DNA, 2µM DS101 primer, 2µM DS102 primer, denature at 94°C for 30 seconds, anneal to 37°C for 30 seconds, extend at 72°C for 60 seconds for 30 cycles, followed by a 72°C soak for 600 seconds, following by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit employing AmpliTaq Thermal Stable DNA Polymerase, total
25 reaction volume 50µL, according to the manufacturer's instructions. The product, 5'-*UBC5*, was analysed by gel electrophoresis and was found to be of the expected size, 229bp. The amplified PCR product was purified

using a Promega Wizard PCR DNA purification kit according to the manufacturer's instructions.

PCR reactions were performed to amplify the 3' end of the *UBC5* gene.

- 5 Conditions were as follows: 1000-10ng/mL S288C genomic DNA, 2 μ M DS103 primer, 2 μ M DS104 primer, denature at 94°C for 30 seconds, anneal to 37°C for 30 seconds, extend at 72°C for 60 seconds for 30 cycles, followed by a 72°C soak for 600 seconds, following by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus
- 10 PCR kit employing AmpliTaq Thermal Stable DNA Polymerase, total reaction volume 50 μ L, according to the manufacturer's instructions. The product, 3'-*UBC5*, was analysed by gel electrophoresis and was found to be of the expected size, 327bp.

- 15 The 5'-*UBC5* DNA fragment was digested to completion with *NotI* and *SalI*, phenol/chloroform extracted and cloned into *NotI/SalI* linearised and phosphatased pBST+ to generate plasmid pDB2262 (Figure 16). The 3'-*UBC5* DNA fragment was digested to completion with *NheI* and *HindIII*, phenol/chloroform extracted and cloned into *NheI/HindIII* linearised and
- 20 phophatased pBST+ to generate plasmid pDB2264 (Figure 17). The DNA inserts of pDB2262 and pDB2264 were sequenced to confirm their identity. Plasmid pDB2264 was digested to completion with *HindIII/NheI* and the 327bp fragment corresponding to the 3' end of *UBC5* isolated and cloned into pDB2262, linearised with *HindIII/NheI* and phophatased. The
- 25 resultant plasmid called pDB2275 contained the 5' and 3' ends of the *UBC5* gene, separated by a unique *HindIII* site (Figure 18). The entire genomic *URA3* gene isolated as a 1.2kb *HindIII* fragment was cloned into linearised pDB2275 with *HindIII* and phosphatased, generating plasmids

pDB2276 (Figure 19) and pDB2277 (Figure 1) which only differed from each other by the orientation of the *URA3* marker gene.

DS569 *ura3* [pAYE329] was transformed to uracil prototrophy with the
5 5'-*UBC5-URA3-UBC5*-3' disrupting fragment isolated from either
pDB2276 or pDB2277 as 1.7kb *MluI-XbaI* fragments. The rHA
productivity of these yeast transformants was assessed in 10mL shake
flask culture. Yeast were inoculated into YNB (Difco) minimal medium,
buffered with sodium phosphate/citrate pH 6.0 and containing 2% w/v
10 glucose, and incubated at 30°C, 200 rpm for 3 days. The rHA
productivity was estimated by rocket immunoelectrophoresis against HSA
standards (25-150µg/mL). The rHA productivity of DS569 [pAYE329]
under these conditions was calculated to be approximately 40mg/L, while
the rHA productivity of some of the pDB2276 or pDB2277 transformants
15 measured at the same time was increased to a level greater than that of
DS569 [pAYE329], calculated to be approximately 60mg/L (Figure 2).

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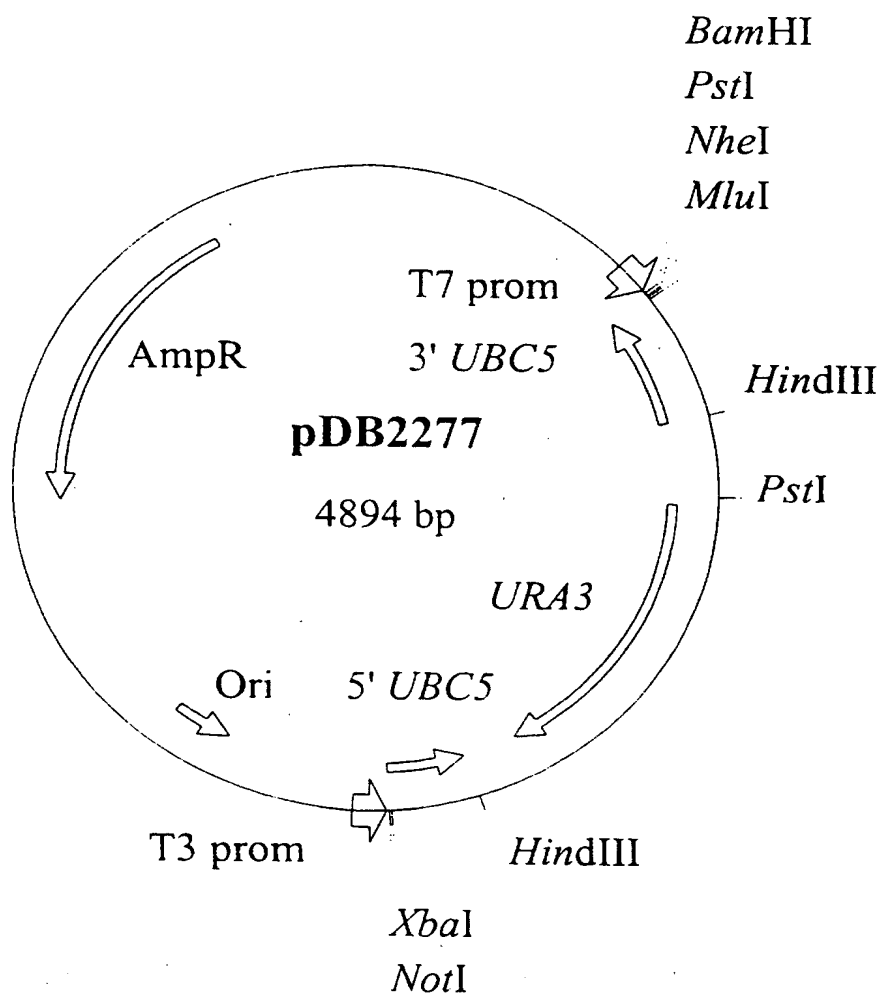
CLAIMS

1. A process of producing a fungal cell derived product, comprising
 - (i) providing a fungal cell having a plasmid, the plasmid
5 comprising a functional coding sequence for a protein, and the fungal cell having a modified level of Ubc4p or Ubc5p activity, and
 - (ii) culturing the cell to produce the fungal cell derived product.
2. A process according to Claim 1 wherein the fungal cell derived
10 product is a desired protein encoded by the said coding sequence, the said modified level of Ubc4p or Ubc5p activity is lower than normal for the cell, and the copy number of the plasmid is increased.
3. A process according to Claim 2 wherein the cell has a substantially
15 zero Ubc4p or Ubc5p activity.
4. A process according to Claim 2 or 3 wherein the cell produces a compound which interferes with the binding of the Ubc4p or Ubc5p encoded product to its target protein.
20
5. A process according to Claim 2 or 3 wherein the *UBC* gene is modified such that substantially no protein is produced therefrom or such that any protein produced therefrom has a reduced level of Ubc4p or Ubc5p activity.
25
6. A process according to Claim 5 wherein at least part of the *UBC4* or *UBC5* gene is deleted.

7. A process according to Claim 5 wherein the *UBC4* or *UBC5* gene is modified such that the ubiquitin-accepting cysteine in any protein produced from the said gene is absent or of reduced ubiquitin-accepting activity.
- 5 8. A process according to Claim 5 wherein the *UBC4* or *UBC5* gene is modified such that any protein produced from the said gene is of reduced ubiquitin-donating activity.
- 10 9. A process according to Claim 5 wherein the *UBC4* or *UBC5* gene is modified such that any protein produced from the said gene is of reduced activity as a result of a mutation of one or more of the first 21 amino acids at the N-terminus of the protein.
- 15 10. A process according to Claim 9 wherein the *UBC4* or *UBC5* gene is modified such that any protein produced from the said gene is of reduced activity as a result of a mutation in the first α -helix (residues 3-13) of the protein.
- 20 11. A process according to Claim 9 wherein the *UBC4* or *UBC5* gene is modified such that any protein produced from the said gene is of reduced activity as a result of a mutation to the glutamic acid at position 10 in the primary sequence.
- 25 12. A process according to Claim 11 wherein the *UBC4* or *UBC5* gene is modified such that any protein produced from the said gene is of reduced activity as a result of a lysine or arginine amino acid substitution at position 10 in the primary sequence.

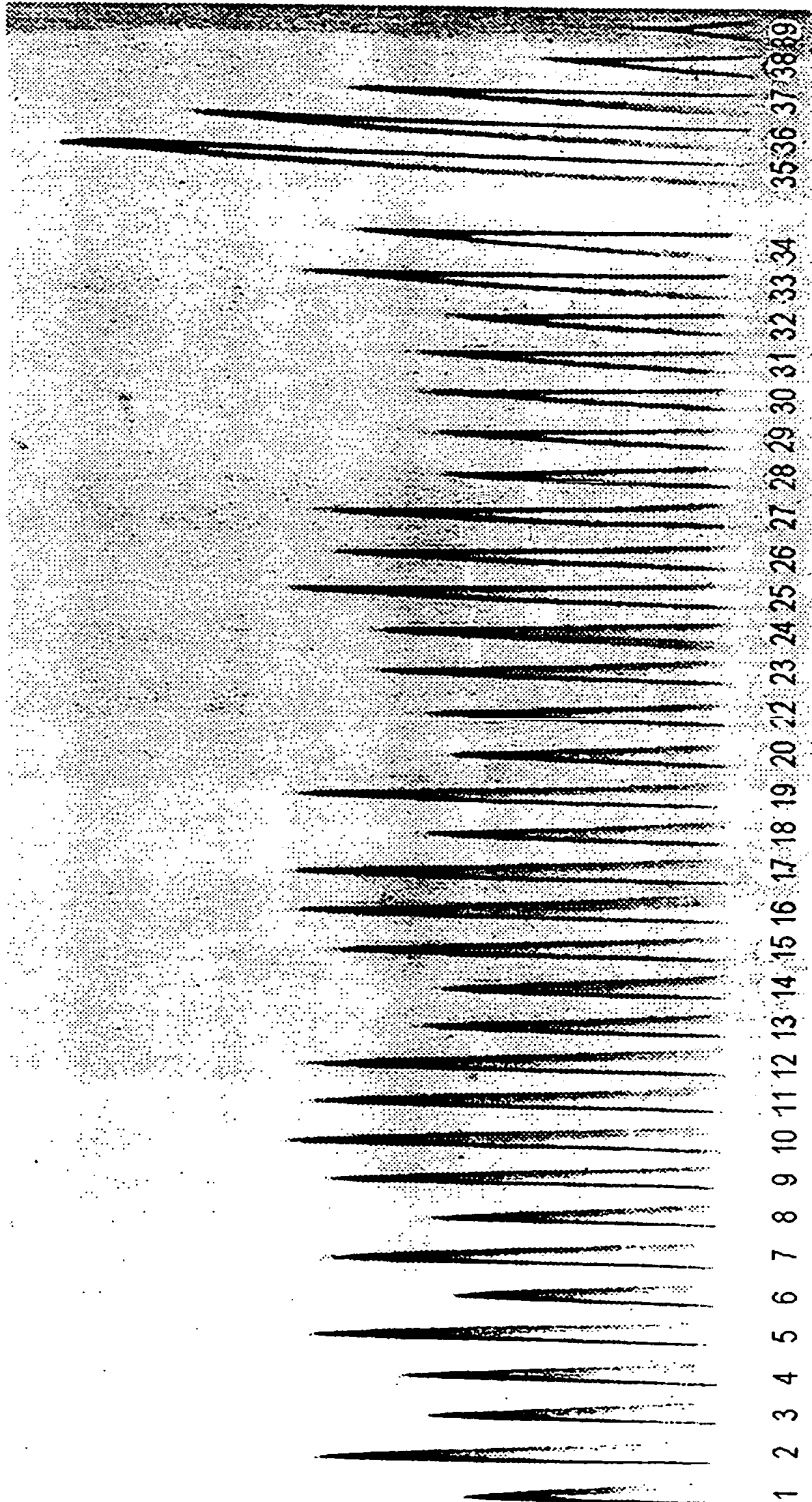
13. A process according to Claim 2 or 3 wherein the cell produces *UBC4* or *UBC5* antisense mRNA.
- 5 14. A process according to any one of Claims 2 to 13 wherein the desired protein is not endogenous to the species to which the cell belongs.
15. A process according to Claim 14 wherein the protein is isolated from the cell culture.
- 10 16. A process according to Claim 14 or 15 wherein the desired protein is a plant or animal protein.
17. A process according to Claim 16 wherein the desired protein is a
15 human protein.
18. A process according to any one of the preceding claims wherein the fungal cell is *Saccharomyces cerevisiae*.
- 20 19. A process according to Claim 18 wherein the plasmid is a 2 μ m-based plasmid.
20. A desired protein produced by a process according to any one of Claims 2-19.
- 25 21. The use of a means to vary Ubc4p or Ubc5p activity in a fungal cell to control the copy number of a plasmid in that cell.

Figure 1



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Figure 2



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Figure 3

CTGCAGTACTCTTTTGATTCTGTAGGAAACCAGCGAAGAACGTACTCTTGCCTGA

*Pst*I

AGAGAAGTTTTTTTTATTTATTTATATTTTGTTCTGGAAGCTCTTTACAGAATGGA

GTAGGAAAATATATAGAGAGGAAAAGCGAAATCGTTACGAGAATAAATAATCAA

GAAAAGAACTTGAACTTGGCTTTTCCAAAACAACAGAAGTAGCGTTAATTTACT

TTCACCGTAAAATTCAACTCTTTAAATATAGTCCACTTAGTAAATTCTTGCCAATT

TGCATGATAAATTCGAACCCATTCTCTCAAATAAAGGGTCCTCATAATTCCATG

GAAAGAAAGTTTTCTTGAACATTAAGAATAAAAAGGCCAAAAAAGAAAAAAAAAAAA

GCACAGCTACTGTTTTAGTCAACATTCCTTTCTCACTGGAATGCACAAGGTGTCAT

TCCTGAACAAGGGTAACTGCACTATTCATATGTCCACCTTATGACTTCATAAAAA

GTTTGACAATAAGTAGTCTTACGTGATAAGAAATGATGTAACATAAGGCTAATGT

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CAAATATCAATGCCAATGGTAACATCTGTCTGGACATCCTAAAGGATCAATGGTC

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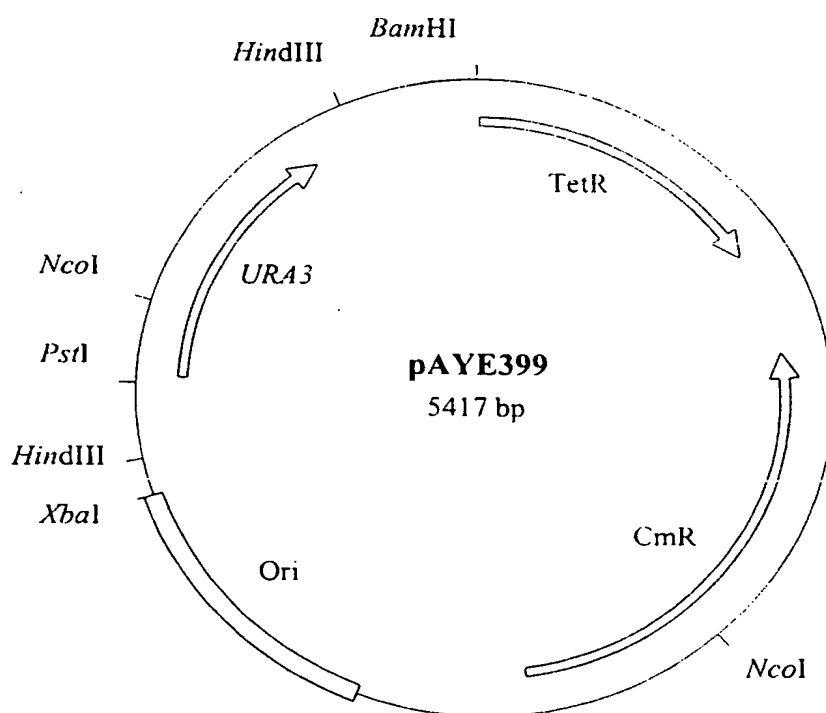
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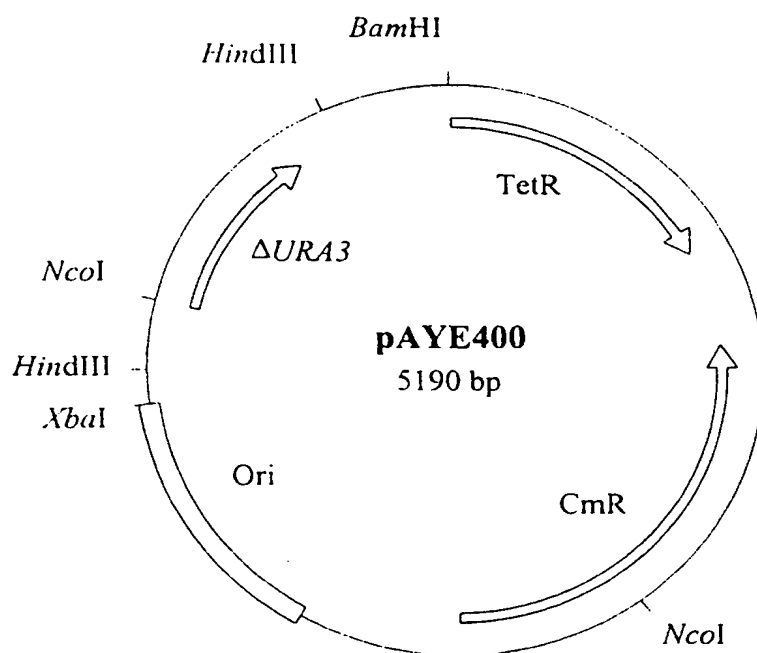
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Figure 4

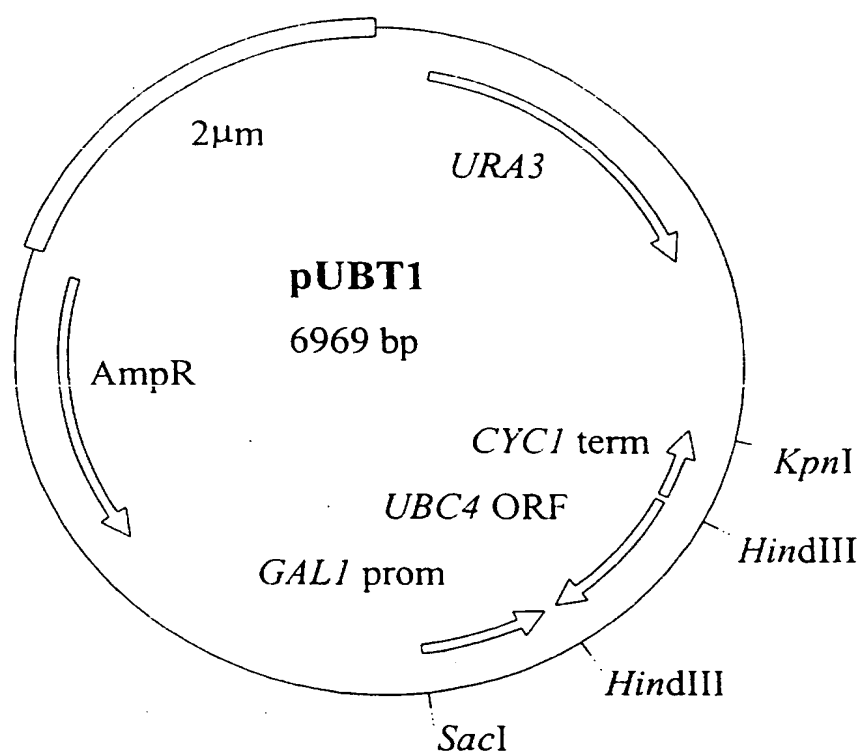
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Figure 5



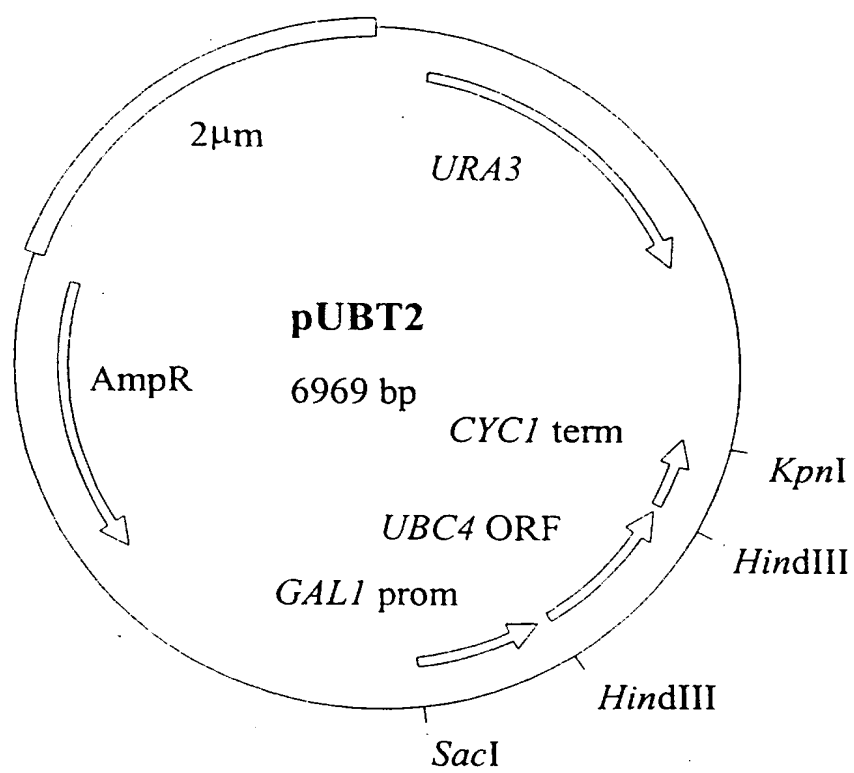
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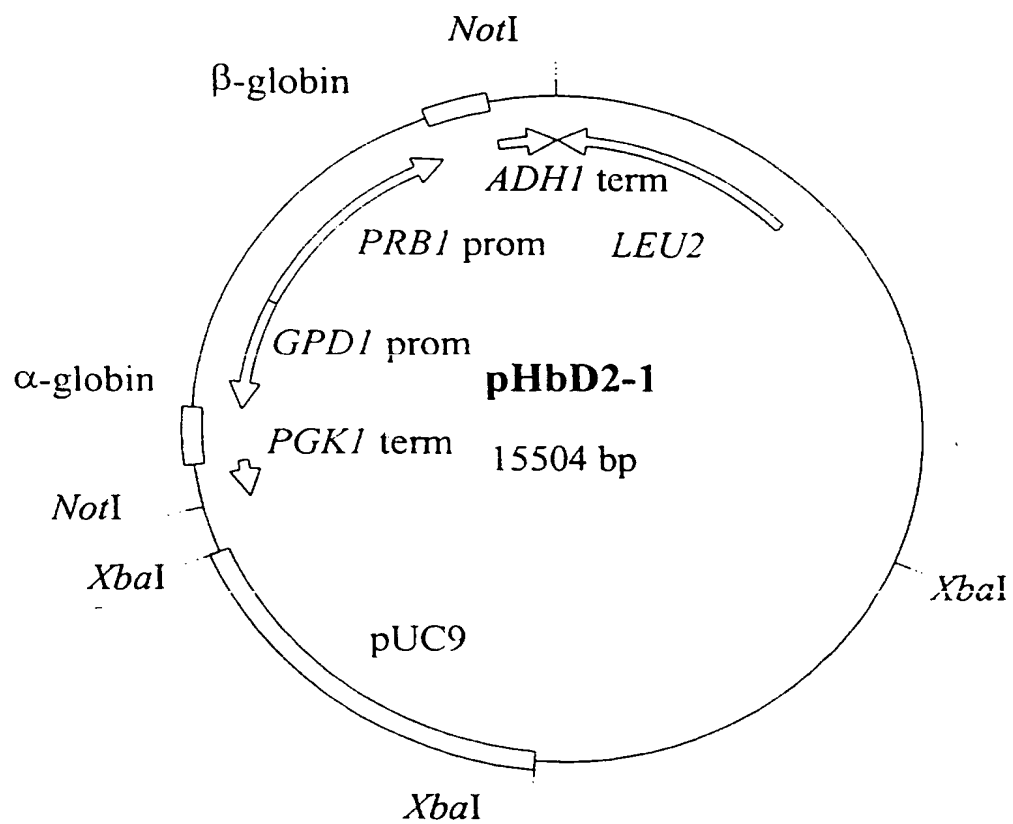
Figure 6



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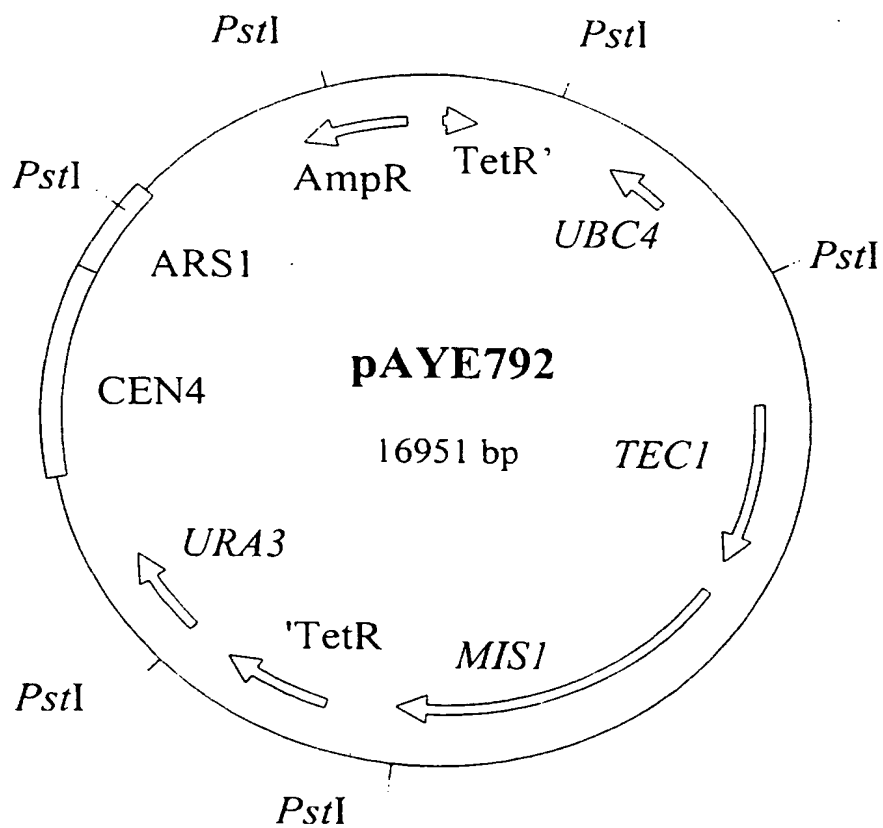
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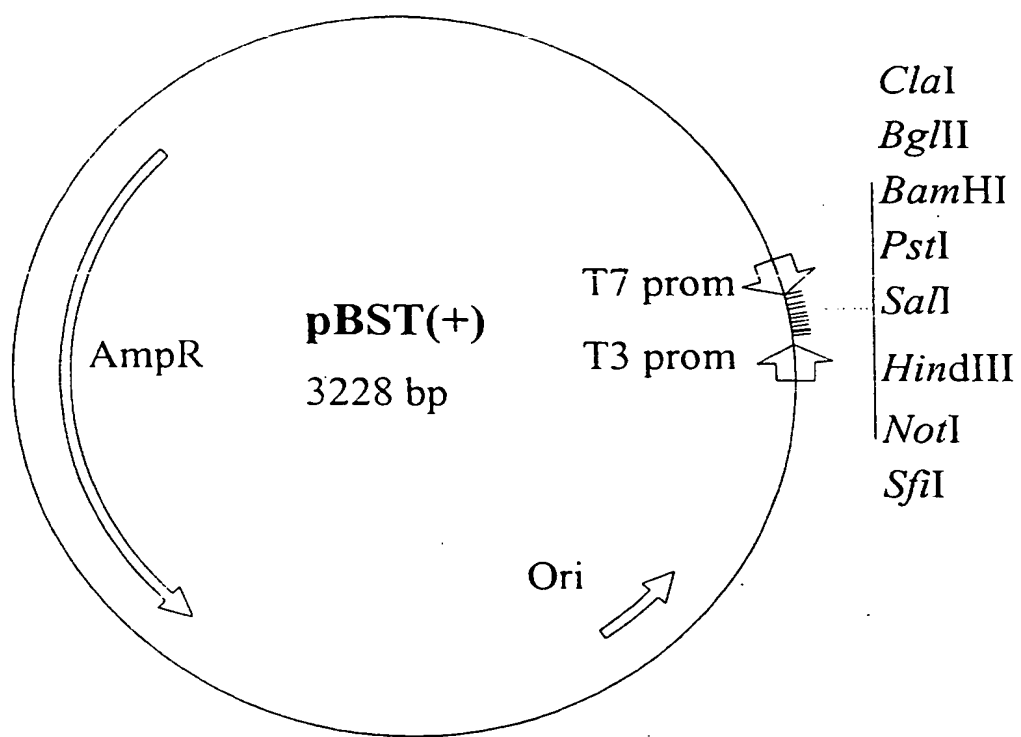
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Figure 8

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Figure 9

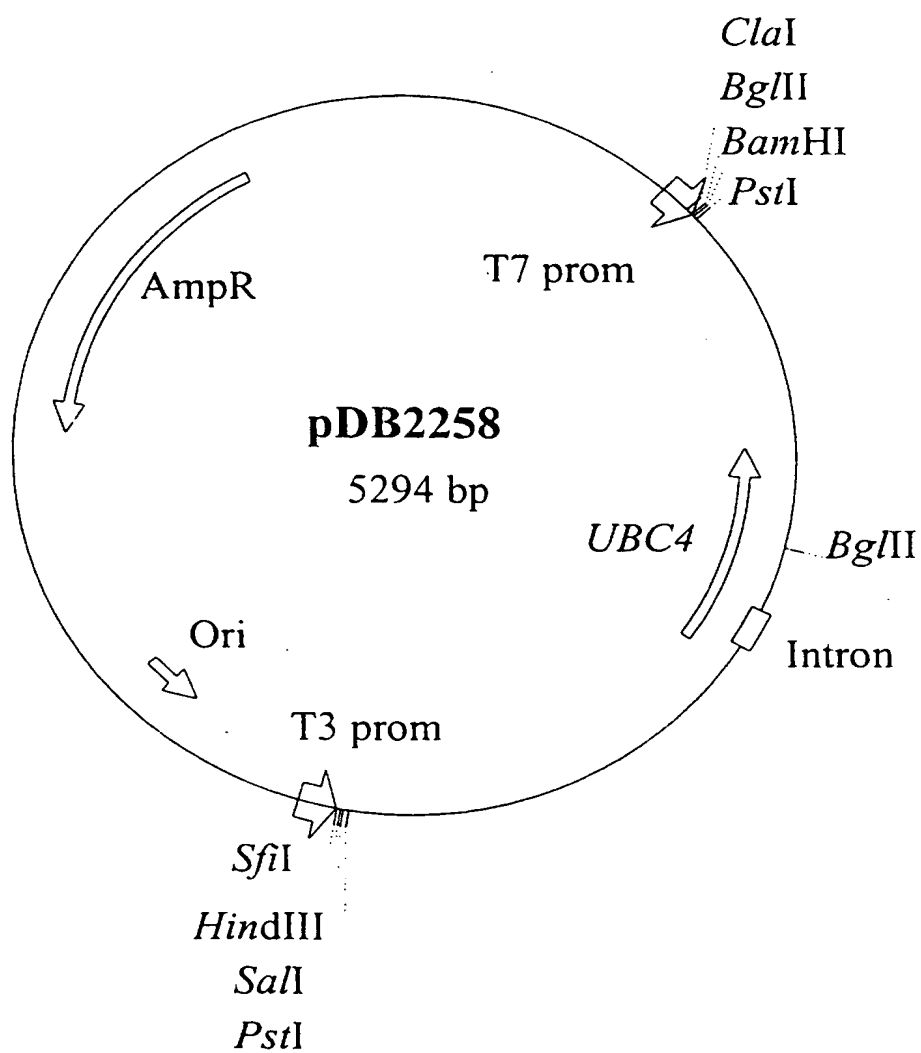


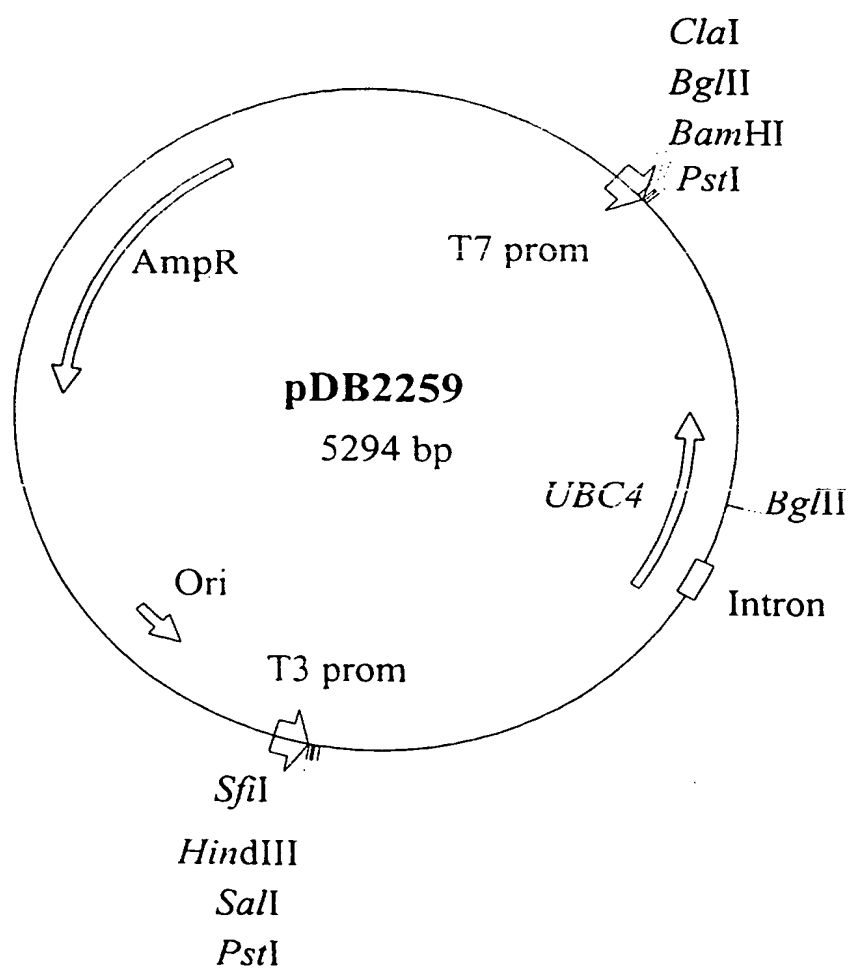
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Figure 10



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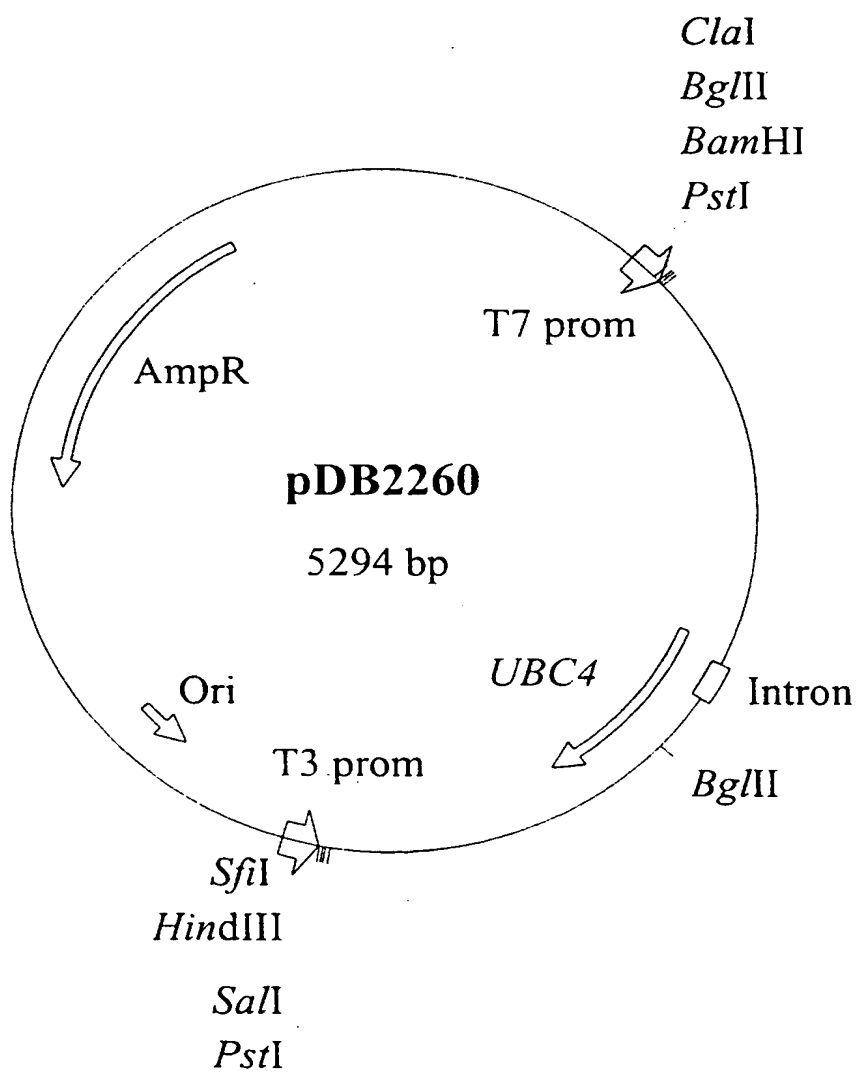
Figure 11



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Figure 12

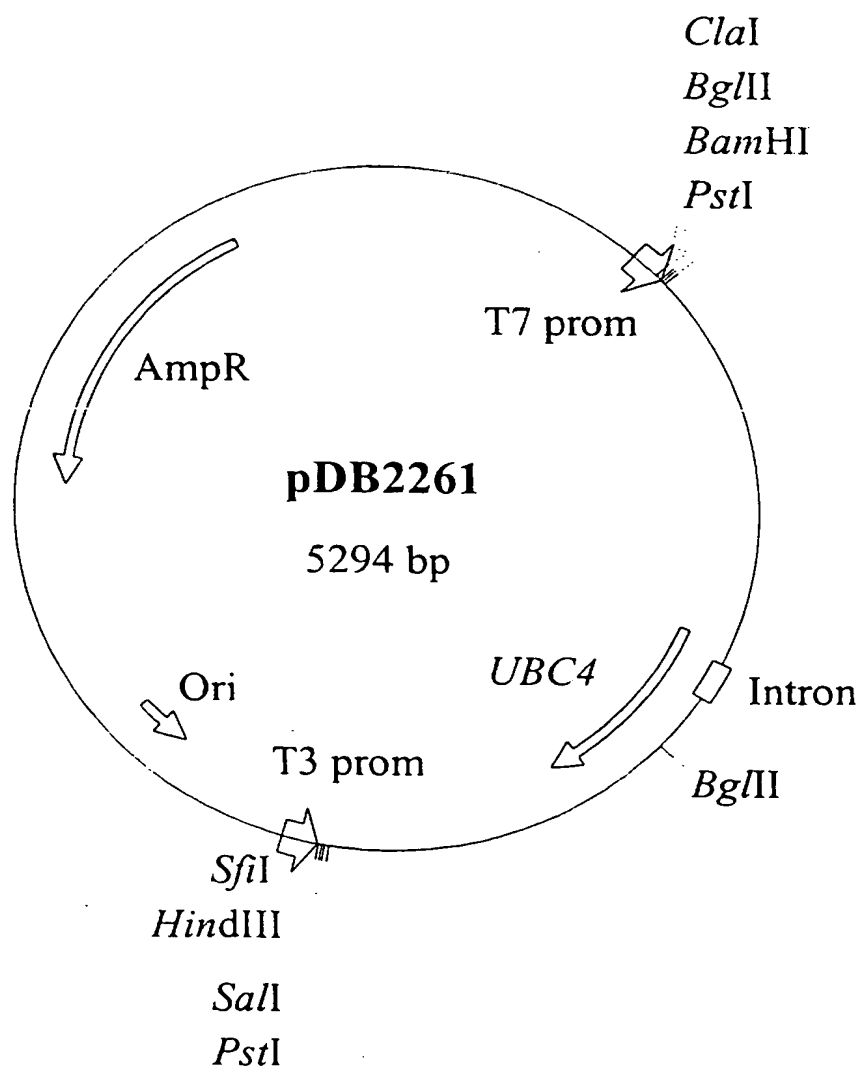
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Figure 13



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Figure 14



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Figure 15

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*Bgl*II

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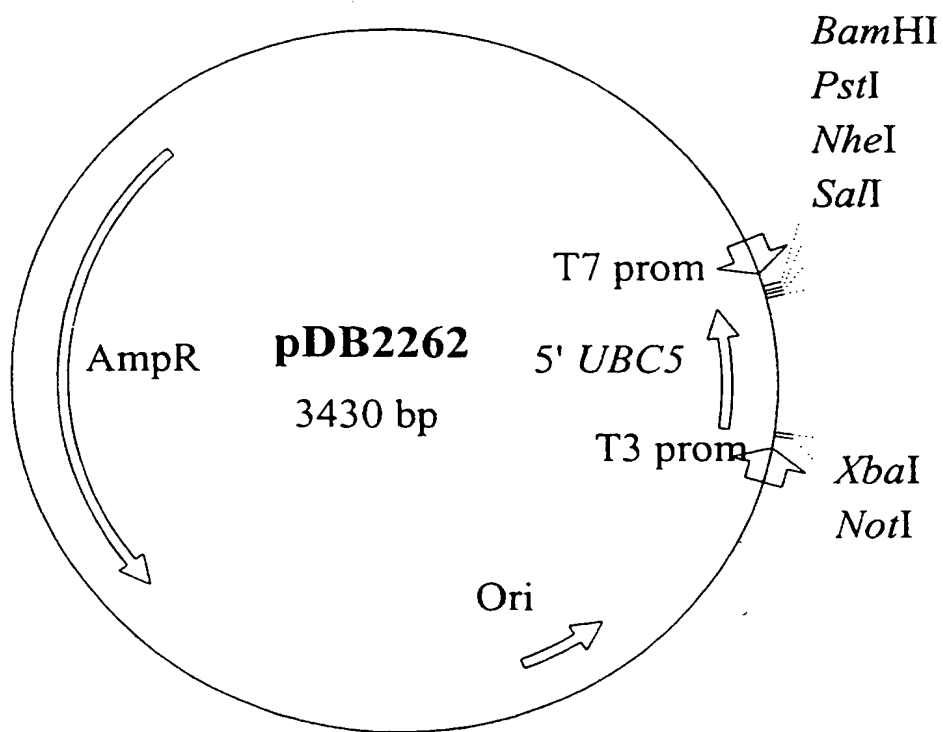
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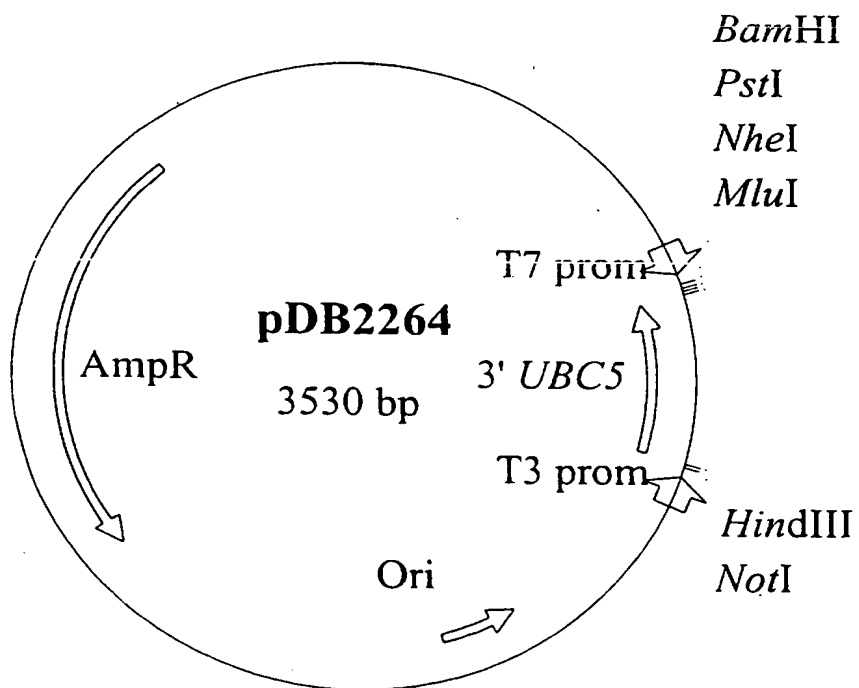
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Figure 16



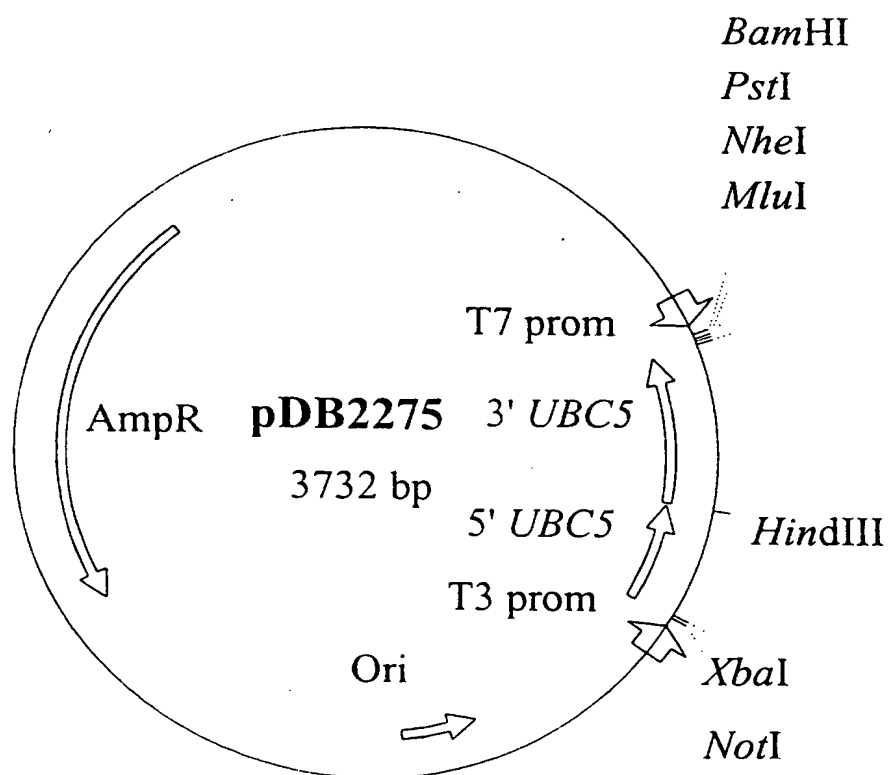
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Figure 17



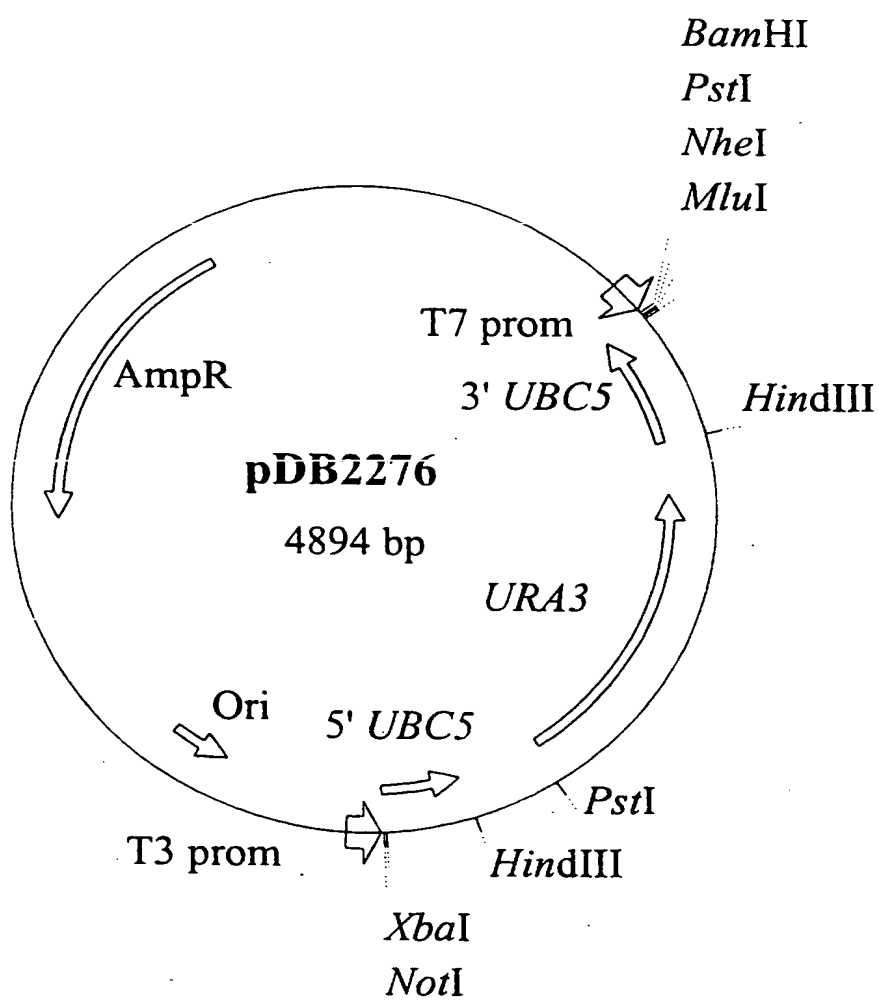
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Figure 18



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Figure 19



INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/01885

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/52 C12N9/00 C12N15/81 C12N15/11 C12N1/19
 //(C12N1/19,C12R1:865),C12N15:69

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BETTING J AND SEUFERT W: "A yeast Ubc9 mutant protein with temperature-sensitive in vivo function is subject to conditional proteolysis by a ubiquitin- and proteasome-dependent pathway" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 271, no. 42, 18 October 1996, pages 25790-25796, XP002085128 see abstract and figure 9 ---	1-8,13, 18-20
A	SLEEP D ET AL: "Saccharomyces cerevisiae strains that overexpress heterologous proteins" BIO/TECHNOLOGY, vol. 9, February 1991, pages 183-187, XP002085129 cited in the application --- -/--	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

24 November 1998

Date of mailing of the international search report

08/12/1998

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INTERNATIONAL SEARCH REPORT

Inter. Appl. Application No

PCT/GB 98/01885

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 12045 A (CHIRON CORP ;BISHOP ROBERT J (US); INNIS MICHAEL A (US)) 3 April 1997 ---	
A	SEUFERT W AND JENTSCH S: "Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins" EMBO JOURNAL., vol. 9, no. 2, 1990, pages 543-550, XP002085131 cited in the application ---	
A	COOK W WT AL: "Tertiary Structures of Class I Ubiquitin-Conjugating Enzymes Are Highly Conserved: Crystal Structure of Yeast Ubc4" BIOCHEMISTRY, vol. 32, 1993, pages 13809-13817, XP002085132 cited in the application ---	
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